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MAST CELL CHANGES IN INH-INDUCED NEUROPATHY IN THE RAT

By

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Received 22 vi.66

The normal occurrence of mast cells in various compartments of peripheral nerves in several animal species has been clearly demonstrated (Torp 1961, Enerback *et al* 1965)

Recent experimental studies have revealed that mast cells in peripheral nerves participate in the tissue reactions occurring in sectioned and crushed peripheral nerves (Gamble & Goldby 1961, Boschi 1964, Enerback *et al* 1964, 1965, Olsson 1965). At the site of the primary lesion in sectioned and crushed sciatic nerves of the rat we have found an early degranulation of mast cells. Furthermore, during the first weeks after nerve section there was a marked increase in the number of endoneurial mast cells throughout the distal degenerating part of the sciatic nerve (Enerbäck *et al* 1964, 1965, Olsson 1965).

Neuropathy induced by isonicotinic acid hydrazid (INH) provides an opportunity for further investigations of mast cells in pathological conditions of the peripheral nervous system of the rat. Soon after the application of INH in the treatment of tuberculosis, several reports described functional disturbances of peripheral nerves (Heilmeyer *et al* 1952, Hook 1953, Bunge & Schultz Ehlbeck 1953, Fust 1953, Jones & Jones 1953, Lubing 1953, Biehl & Nimitz 1954, Biehl & Viller 1954, Klinghardt *et al* 1954, Oestreicher *et al* 1954, Fust *et al* 1955). Morphological lesions of peripheral nerves have also been produced experimentally by feeding laboratory animals with high doses of INH (Klinghardt 1954, Zbinden & Studer 1955, Schlaepfer & Hager 1964 a, b, c). In the recent light and electromicroscopic investigation by Schlaepfer & Hager the peripheral nerve lesions were characterized as a primary axonal degeneration with a subsequent breakdown of the myelin sheaths. Later there were regenerative phenomena of axons and myelin sheaths and a mild endoneurial fibrosis. However, participation by mast cells in this peripheral neuropathy has not yet been described.

The following study was undertaken in order to find out whether the INH induced neuropathy of the rat is associated with mast cell changes in the damaged nerves.

MATERIAL AND METHODS

36 Sprague Dawley rats of both sexes were used. The rats weighed between 150 and 250 grams at the beginning of the experiments. They were kept on a diet of commercial rat pellets and water ad libitum.

Under light ether anesthesia, 350 mg/kg body weight isonicotinic acid hydrazide¹ dissolved in Ringer solution was given by oral intubation daily for two weeks, with the exception of one group of rats, which were sacrificed after treatment for one week. Control rats received Ringer solution by oral intubation.

Groups of three rats were sacrificed by decapitation 1, 3, 4, 6, 10 and 16 weeks respectively after the first dose.

The two sciatic nerves were taken for histological examination. From rats with observation times of 4, 10 and 16 weeks the following tissues were taken at well dorsal and ventral spinal nerve roots, dorsal root ganglions, sciatic plexus, common peroneal nerve, tibial nerve, sural nerve.

The tissues were fixed in an acetic acid formalin mixture for 24 hours (Enerbäck 1966). Longitudinal paraffin sections, 5 microns thick, from varying levels of the nerves were stained in a 0.5 per cent water solution of toluidine blue in a citric acid disodium phosphate buffer according to Mellman, with pH adjusted to 4.5. The sections were rapidly passed through alcohol to xylol and mounted in canada balsam.

Myelin sheaths were stained by the luxol fast blue, cresyl violet method of Kläver & Batters (1953) and Palmgren's silver impregnation technique was used for axons (Palmgren 1949).

RESULTS

All experimental animals showed diminished motor activity during the last days of the treatment but paralysis was never observed. There was also a loss of body weight. These general signs disappeared rapidly when treatment was suspended.

Mast Cells in Nerves of Control Rats

The control rats displayed the same distribution and morphology of mast cells in peripheral nerves as in the normal rats described elsewhere (Enerbäck, Olsson & Sourander 1965). Therefore only a brief summary will be given here.

The epineurium and perineurium of all peripheral nerve trunks examined displayed numerous mast cells around blood vessels. Scattered mast cells occurred between the nerve fibres in the endoneurium of the nerve fasciculi. The endoneurial mast cells were never seen in groups but appeared fairly uniformly distributed in transverse sections of the nerves. The cytoplasm was most often elongated along the line of the nerve. The cytoplasmic granules of the mast cells showed a strong metachromasia that was resistant to alcohol dehydration in specimens stained with toluidine blue. No mast cells were identified in dorsal and ventral spinal nerve roots. In dorsal root ganglions, mast cells were found in the capsule and occasionally in the ganglion-nerve junction but never within the ganglions.

¹ Isoniazid (® INH) obtained from ACO Stockholm Sweden

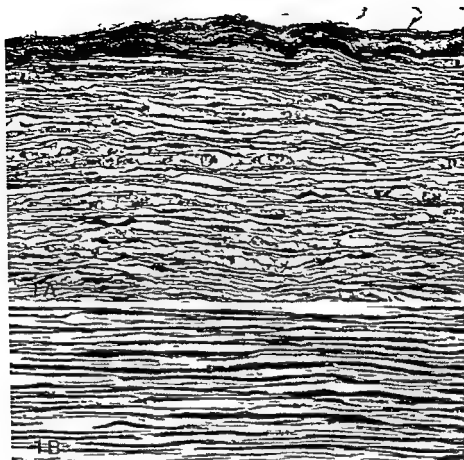


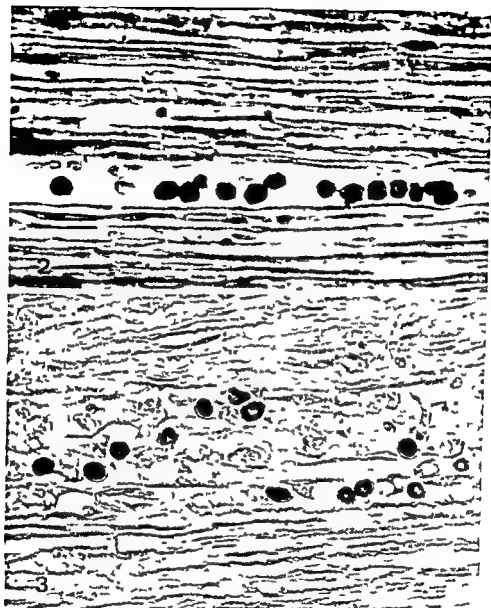
Fig 1

A Marked degenerative changes of axons in the sciatic nerve of a rat treated with INH for fourteen days B Normal appearance of axons from a control rat
Silver impregnation (Palmgren)

Nerve Fibre Changes in INH-Treated Rats

The earliest changes were observed in axons, after treatment for one week with INH. Focal fragmentations and varicosities of some axis cylinders were found in silver impregnated specimens of the sciatic nerve in all the rats. Such lesions were much more advanced after treatment for 14 days; there also seemed to be a marked reduction in the number of axons (Fig 1). In the latest stages, however, such axonal changes were much less pronounced.

In contrast to the axonal changes observed after treatment for one week, only slight changes of myelin sheaths could be detected in adjacent specimens stained by the luxol fast blue-cresyl violet technique of Klüver & Barrera. These animals displayed only a few varicosities and fragmentations of myelin sheaths. After treatment for two weeks,



Figs 2 3

Fig 2 Demyelination and accumulation of mast cells in the demyelinated part of a sciatic nerve from an INH treated rat *Kiliter & Barrera*

Fig 3 Focal nerve fibre lesion associated with an accumulation of small rounded endoneurial mast cells in the sciatic nerve of an INH treated rat. Phase contrast micrograph of a toluidine blue stained specimen

all rats showed marked demyelination, often with focal areas exhibiting varicosities and fragmentation of the myelin sheaths (Figs 2 3). Rats with observation times exceeding ten weeks presented no clear changes in myelin sheaths of the sciatic nerves

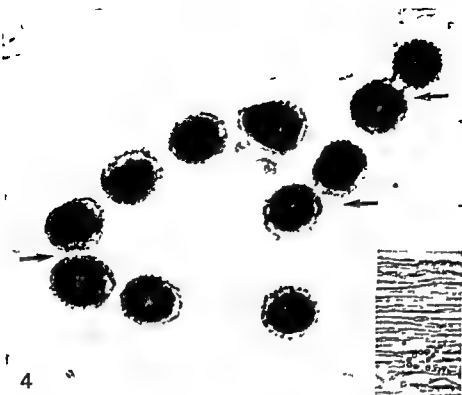


Fig 4

Cluster of mast cells in the vicinity of an endoneurial blood vessel in the sciatic nerve of a rat treated with INH. Note the almost identical appearance of the sparsely granulated mast cells and cytoplasmic bridges between adjacent cells (arrows). Toluidine blue.

In the damaged areas, mitosis of Schwann cells was occasionally found in the first two groups of rats. Later Schwann cells formed so called B ngner's band in the damaged parts.

Changes in axons, myelin sheaths and Schwann cells were most pronounced in the sciatic nerve and its branches, e.g. the common peroneal, tibial and sural nerves. In dorsal and ventral nerve roots nerve fibre lesions were extremely rare. In dorsal root ganglions, a few ganglion cells displayed lysis of their Nissl bodies and fragmentation of some axon cylinders.

Mast Cell Changes in Peripheral Nerves of INH Treated Rats

The earliest morphological changes in mast cells were found in the sciatic nerve of rats given INH for one week. Parts in and adjacent to areas showing degenerative changes of nerve fibres had scattered endoneurial mast cells with special morphological characteristics. These cells had a rounded nucleus, which occupied the central part of the cell.

doxine deficiency (*Oestreicher et al* 1964). There are also observations that deficiency in pyridoxine may be associated with a peripheral neuropathy (*Spies et al* 1939 *Voller et al* 1953 *Swank & Adams* 1945 *Viclor & Adams* 1956).

The present findings of endoneurial mast cell changes associated structurally and temporally with degenerative changes of axons and myelin sheaths and the absence of such mast cell changes in the epineurium suggest that this mast cell reaction is secondary to the nerve fibre lesions.

It has previously been shown that similar morphological and numerical changes of endoneurial mast cells occur in the distal part of sectioned and crushed nerves of the rat (*Enerbaek et al* 1964 1967 *Olsson* 1965). The present finding of similar endoneurial mast cell changes in a metabolically induced peripheral neuropathy strengthens the hypothesis that the endoneurial mast cell reaction is elicited from the damaged nerve fibres.

SUMMARY

A study was made on the behaviour of mast cells in peripheral nerves in INH induced neuropathy of the rat. The main findings were as follows:

1. Marked lesions of axons and myelin sheaths in the sciatic nerve and its main branches. In spinal nerve roots nerve fibre lesions were extremely rare.

2. In conjunction with degenerative changes of axons and myelin sheaths in the peripheral nerves there was an increasing number of small sparsely granulated mast cells which often appeared in pairs or clusters of cells of almost identical appearance. Occasionally small cytoplasmic bridges were detected between adjacent cells. In the latest stages the numerous endoneurial mast cells were of about the same form and size as in control nerves. In contrast no mast cell changes were seen in the epineurium. Nor could mast cells be found in the spinal nerve roots of INH treated or control rats.

These observations suggest that there is a new formation of endoneurial mast cells in this metabolically induced peripheral neuropathy of the rat. This finding supports the assumption that the numerical increase of endoneurial mast cells in the distal part of sectioned or crushed nerves is elicited from the degenerated nerve fibres.

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ANTI-NUCLEAR FACTORS IN HEPATIC CIRRHOSIS

*With Special Reference to the Different Nuclear Antigenicity of
Various Tissues*

By

VIGGO FABER and PREBEN ELLING

Received 12 April 1966

Sera from patients with hepatic cirrhosis often contain antibodies against various tissue components, and anti nuclear factors (ANF) as determined by the immunofluorescence technique are found with a positive frequency varying from 12 per cent to 44 per cent (7, 12, 13, 18). These differences are probably mainly due to the composition of the series of patients investigated, but some of them may also arise from the use of different nuclear antigens for the studies. Some different ANF reactivities have been reported before. Thus, *Hijmans et al* (14) suggested the presence of at least two antigen-antibody systems, human sera with a very limited nuclear reactivity have been found by different authors (5, 9, 10, 11, 16, 19), tissue-specific ANF has been produced in animals (2, 15), and evidence of granulocyte specific ANF has been presented (2, 3, 8).

When sera from patients with hepatic cirrhosis were examined for the presence of different auto antibodies, it was observed that some sera giving positive reaction for ANF with some tissues as antigen, were completely negative with other tissues. Since the variations found did not seem to be explained by technical circumstances only, and the findings might suggest different organ specificity of the anti nuclear factors, it was decided to extend the investigation. The present report gives the results of determining anti nuclear factors using the immunofluorescence technique with 8 different sources of nuclei as antigen.

MATERIAL

Sera from 96 patients with hepatic cirrhosis collected during the years 1959-63 were investigated. All patients showed clinical and biochemical signs of chronic

Part of this study was presented before the 98th meeting of the Danish Society of Pathology Copenhagen Denmark August 29 1965 and before the First Congress of the European Society of Pathology Warsaw June 1 1966.

This report was aided by grants from *The Daell Fond* and *Konsul Fhrensriek Olesen og hustru f. ni*

liver damage. In 83 of the cases the diagnosis was confirmed by biopsy or at autopsy. The sera that had been stored at 20° C for several years had been used previously for other serological studies (6). Fifty nine of the patients were women, 37 were men. Twenty six of the female and 5 of the male patients were known to have had epidemic hepatitis. Twenty four of the male and 3 of the female patients were known to have been heavy drinkers. More than half of the patients were between 50 and 70 years of age. Twenty five of the 96 patients had died.

Control sera from individuals matched for sex and age were partly derived from healthy donors under 65 years of age (72 sera) and partly from surgical departments from patients over 65 years old with a negative Wassermann reaction (24 sera).

METHODS

The two layer indirect immunofluorescence technique was performed as described in a previous paper (7) using undiluted sera.

Antigens. The following human tissues were employed as antigen: *thyroid gland* from a patient with thyrotoxicosis; *liver* tissue from a patient with secondary biliary cirrhosis; *mucosa of the stomach* from a patient with a duodenal ulcer; *striated muscle* from the same patient; and normal *renal cortex* adjacent to a renal tumour. These tissues were all obtained from the operation theatre, cut into pieces and frozen at -70° C within 15 minutes from the time of removal of the tissues. The tissues were stored at -70° C until use. In addition *blood smears* from peripheral blood of healthy individuals of blood group O were dried with a fan and used unfixed within 15 minutes after their preparation. Finally the *adrenal gland* from a monkey (guenon) was used as antigen prepared in the same way as described for the human tissues above. No fixation was ever used.

All pathological sera were investigated at least twice with intervals of weeks or several months using the different human tissues as antigen. Discrepant results occurred in few cases. Such sera were retested as were sera showing faint or doubtful nuclear fluorescence until confident results were achieved. Appropriate control slides without serum and slides with ANF negative and positive control sera were included in all experiments.

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wl

conjugate which was absorbed with acetone dried guinea pig liver powder never showed non specific anti nuclear reactivity during daily use for nine months.

Microscope

The microscope was a Leitz Ortholux (Zerniche) with equipment for combined fluorescence and phase microscopy with a high pressure mercury vapour lamp (Osram HBO 200) and LG1 exciter filter and a colourless UV absorbing filter.

RESULTS

The results are presented in the Tables 1 and 2 and in fig. 1. As seen from Table 1 only 11 of the pathological sera combined with nuclei of striated muscle, one fourth of the 96 sera reacted with the nuclei of liver tissues, gastric mucosa and those of neutrophilic granulocytes of peripheral blood while about one third of the sera showed a positive reaction for ANF when thyroid gland or small lymphocytes from peripheral blood were used as antigens. With monkey adrenal gland almost three fourths of the sera gave positive ANF reaction.

Among the control sera none reacted with the nuclei of striated muscle. 4/12 sera showed positive ANF reaction with the other human

tissues, while 16 sera combined with the nuclei of monkey adrenal glands

Of the positive reactions with the human tissues 85-90 per cent were given by sera from female patients. Thus 17-50 per cent of all female and 3-22 per cent of all male patients contained anti nuclear factors in their sera. This preponderance of the female sex was not so distinct when guenon tissue was used as antigen, since two thirds (24 out of 37) of the male as compared to three fourths of the female patients (46 out of 59) showed positive ANF reactions.

TABLE 1

Positive Frequency of ANF in 96 Cirrhotic Sera and 96 Control Sera Using 7 Different Human Tissues and One Animal Tissue as Antigen

Number of sera	Striated muscle	Renal cortex	Human tissues				Thyroid gland	Lymphocytes	Monkey tissue
			Liver	Gastric mucosa	Neutrophilic granulocyte	Adrenal gland			
96 patient sera									
Female 59	10	22	23	23	21	31	28	46	
Male 37	1	2	2	3	5	4	8	11	
96 control sera									
Female 59	0	6	7	4	3	8	4	13	
Male 37	0	1	2	2	1	4	1	3	

From Fig. 1, in which the positive ANF reactions using the different tissues as antigen are placed in groups according to age of patients and controls, it is seen that most of the positive reactions were found in the age groups between 60 and 80 years, regardless of the tissues employed.

The varying incidence of ANF found may simply reflect quantitative differences of antigen present in the nuclei, but since the differences in nuclear susceptibility were unrelated to the titres of ANF, it may also be due to the presence of anti-nuclear factors with limited nuclear reactivity, i.e. organ specific anti-nuclear factors. Table 2 shows an analysis with respect to this point. Fifteen sera had very limited nuclear reactivity since they reacted with only one of the 7 human nuclear antigens. Six of these sera stained the small lymphocytes only, 6 the thyroid nuclei only and 3 the nuclei of the gastric mucosa only. No other ANF with apparent organ specificity were found among the pathological sera, except for one serum which reacted with the nuclei of the eosinophilic granulocytes but not with those of neutrophils, lymphocytes or any other of the human nuclei tested. One serum showed a very strong fluorescence of the nuclei of the arterial walls in the tissues, besides a very faint staining of the nuclei of gastric mucosa as the only other ANF present in this serum specimen.

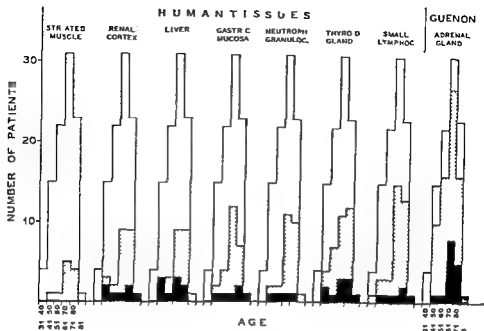


Fig 1

Positivity of ANF in 96 control sera (black columns) and 96 cirrhosis sera (check pattern) in different age groups using 7 human tissues and one animal tissue as antigen

Seven sera contained ANF which reacted with all human tissue nuclei, 28 sera showed positive ANF reaction with more than one but not all human tissues, and altogether 51 (50 + 1) of the pathological sera showed ANF reactivity with one or more human tissues. Forty five (46 - 1) of the pathological sera did not react with any of the 7 different human tissue nuclei.

All sera from cirrhotic patients, except one reacting with one or more of the human tissues, also reacted with the nuclei of the monkey adrenal gland. Twenty-two of the 46 sera not reacting with any of the

TABLE 2

Number of Hepatic Cirrhosis and Control Sera Reacting with One or More Nuclei of 7 Different Human Tissues Employed as Antigen

	Number of human tissues reacting							Total number of positive ANI with one or more tests	Number of negative sera in all tests
	1/7	2/7	3/7	4/7	5/7	6/7	7/7		
Patient sera 96	15	6	3	5	5	9	7	50(+1)*	46(-1)
Control sera 96	4	3	4	1	1	2	0	15	81

* One serum reacted with the nuclei of eosinophilic granulocytes only

human tissues did not react with the nuclei of the monkey adrenal gland either. Twelve of the 15 sera containing apparently organ specific ANF with human tissues gave positive reaction with the nuclei of guenon adrenal gland also.

With regard to the control sera, 4 of them reacted with the nuclei of only one tissue, but not with the other 6 human tissues and 11 sera stained the nuclei of more than one tissue. None of the control sera reacted with all nuclei tested. Eighty one of the control sera did not react with any of the human nuclei tested. Sixteen of the control sera gave a positive ANF reaction with monkey adrenal gland. Among these 16 sera were 12 reacting with one or more of the human tissues and 4 sera showing no other ANF reactivity.

DISCUSSION

The present demonstration of dependence of anti nuclear response on the nature of the tissue employed as antigen shows that the choice of nuclear source may be important when testing sera for anti nuclear reactivity. This applies to the sera from the patients with hepatic cirrhosis and to those from the present control material as well as to sera from patients with myasthenia gravis, as *Feltkamp* (9) has shown and to sera from patients with rheumatoid arthritis (3), but it is probably of little or no importance with sera from patients with disseminated lupus erythematosus possessing greater reactivity with different tissue nuclei (1). The fact that such sera have been used as positive control sera in most of the reports dealing with determinations of ANF in different pathological states may explain why only few recent papers suggest a possibly different antigenicity of nuclei of various tissues.

Though a standardization of all the steps in the technique which is highly necessary in comparative investigations of this kind has as far as possible been carried out, it is still feasible that unnoticed technical differences (*e.g.* in preparation, storage or handling of tissues or sera with a consequent degradation of antigen or antibody) may explain some of the varying results presented. Therefore the findings call for careful attempts to standardize nuclear antigen in addition to the examinations of the significance in the reaction of the composition of conjugates etc. However, the lack of agreement between the reactivity of the different nuclei and the variability in antibody titres besides the identical results repeatedly obtained even with great intervals suggest the existence of antinuclear factors with different organ (and species) specificities. It has been an invaluable help to use phase contrast microscopy, and conventional staining of the immuno fluorescence preparations has sometimes also been necessary to ascertain which nuclei actually were stained. These procedures have in some cases revealed that fluorescent nuclei in sections from cirrhotic livers originated in infil-

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MICROTECHNIQUE FOR QUANTITATIVE MEASUREMENT OF RENIN IN PLASMA

By

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Many methods of measuring renin activity have been described in the last few years *Brown et al* (1964), *Boucher et al* (1964), *Helmer & Judson* (1963), *Pickens et al* (1965) and *Vito & Fasciolo* (1965). With these methods, which demand from 10 to 20 ml of plasma, renin, after total or partial inhibition of the angiotensinase activity, is allowed to react *in vitro* with renin substrate, angiotensinogen, and the angiotensin produced is measured.

The present study describes a microtechnique demanding only 300 μ l of plasma. The total inactivation of angiotensinase is made unnecessary by determination of the reduction of the substrate concentration instead of measuring the angiotensin produced. This principle is generally used in enzymology, and *Munoz et al* (1943, 1946) have used it in the determination of renin in human serum.

METHODS

The renin used was the commercial preparation of NBC (Cleveland Ohio). The substrate used was a plasma pool from albino rats that had been nephrectomized 24 hours earlier. pH was adjusted to 3.6 by 2N of phosphoric acid for 10 minutes at 25 degrees Celsius and subsequently to 7.5 by 6N of NaOH. After this process with the renin preparation used, and the temperature applied with an incubation time of 10 minutes and with the concentration of angiotensin reached no measurable angiotensinase activity was found (*Bing* 1964). On the other hand angiotensinase activity is found by increasing the time of incubation.

1. The Durability of the Substrate

Analyses in duplicate containing 100 μ l of substrate + 100 μ l of trisbuffer pH 7.5 are incubated in siliconized tubes at 37 degrees C for a time varying from zero to 72 hours.

The solution is covered by 600 μ l of a mixture consisting of equal parts of toluene and paraffin oil. Toluene is used as a bacteriostatic; paraffin oil is added to avoid evaporation. After this incubation 800 μ l of a 10 minutes preheated renin solution (Trisbuffer pH 7.5 containing 0.1 Goldblatt Dog Unit (G.U.) per ml) is added to half of the tubes in order to determine the concentration of angiotensinogen and 800 μ l of trisbuffer pH 7.5 is added to the other half of the tubes in order to detect the inactivation of the substrate. The mixture is incubated for

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10 minutes at 37 degrees C. The procedure to follow consists in admixture of 10 ml of 2N HCl and placing the tube in boiling water for 10 minutes. The precipitate is removed by centrifugation and 10 ml of 2N NaOH is added to the supernate which is then stored at -20 degrees C. The concentration of angiotensin in the supernate is determined as described below, and is expressed as nanograms (ng) per ml.

2 Formation of Angiotensin at Varying Substrate Concentrations

Trisbuffer pH 7.5 is added to an amount of substrate ranging from zero to 500 mμl until the total volume is 500 mμl. After preheating of both solutions this mixture is incubated with 500 mμl of a renin solution containing 0.16 G.U. per ml (in Tris buffer pH 7.5). The concentration of renin during the reaction is identical with that used for determination of the durability of the substrate and transforms 100 per cent of the substrate during a 10 minutes' incubation at 37 degrees C. The procedure to follow as described above (1). In order to determine the total substrate concentration this scheme is followed by using 100 mμl of substrate and a renin solution containing 2 G.U. per ml.

3 Measurement of Known Renin Concentrations

A series of 3 identical tubes a, b and c is produced each containing 100 mμl of the same renin solution (in Trisbuffer pH 7.5) and 100 mμl of substrate. The concentration of the renin solution is varied from 0.0001 G.U. per ml to 0.0006 G.U. per ml. The tubes are then incubated for 10 minutes at 37 degrees C. and containing 100 mμl of Trisbuffer pH 7.5 and

37 degrees C. Then tubes a and b are incubated with 800 mμl of a preheated renin solution containing 0.1 G.U. per ml while tubes c and d are incubated for 10 minutes at 37 degrees C. with 800 mμl of preheated Trisbuffer pH 7.5. The procedure to follow as described above (1).

4 Measurement of Renin in Plasma

The rats are sacrificed by heparinized tube which is followed by centrifugation it is submitted to 100 mμl of plasma. section: Measurement of known Renin Concentrations"

5 Biological Assay of Angiotensin

Angiotensin is measured by its pressor effect in rats. Rats weighing 180 g are given a ganglioplegic dose of 0.5 mg/kg of chloral hydrate. They are then injected with 0.5 ml of a solution containing from 1 to 6 ng of angiotensin at 10 minute intervals. The standard solutions of angiotensin are used. The standard solutions are injected until they give almost the same pressor response. The sample to be assayed is given between two standards on either side and this is repeated not less than three times for each sample. From the two standards before and after the unknown sample a linear interpolation is made to the sensitivity at the time of injection of the unknown. The difference if any from this value is counted linearly in the calculation of the concentration.

RESULTS

The Durability of the Substrate

It is a condition of the determination of the renin activity from the reduction of the substrate concentration that the substrate should be

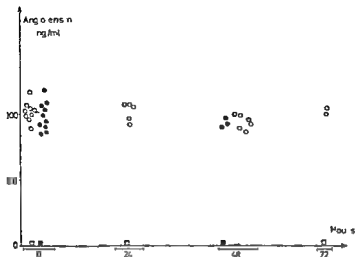


Fig 1

The marks ○ and ● indicate the durability of two substrate pools after incubation for up to 72 hours at 37 degrees C. The marks □ and ■ indicate that pressor substances are not produced

free from renin and durable in the experimental conditions. In order to get a substrate free from renin, plasma from rats nephrectomized 24 hours earlier was employed. The concentration of angiotensinogen in such plasma is 10 times as high as in normal plasma (Bing 1964).

If the substrate is placed as described in Methods 1 from 0 to 72 hours at 37 degrees C, the substrate concentration is found not to have changed (Fig 1). In addition, it is seen from Fig 1 that by being stored the substrate does not produce any pressor substances. Consequently, the substrate is free from renin, stable, and not digested by proteolytic enzymes in the time used. (After being stored at -20 degrees C for a long time (3 months), one of the substrates was decomposed during the incubation and an angiotensin-like pressor substance was produced. In the analysis used, a tube 4 is included to certify that this has not happened.)

Angiotensin Produced at Varying Substrate Concentrations

If the concentration of renin is kept constant and the concentration of the substrate is varied as mentioned in Methods 1, concentrations of angiotensin are found after 10 minutes incubation as shown in Fig 2. The concentration of angiotensin is seen to vary in proportion to the concentration of the substrate. The concentration of angiotensin produced corresponds to a 30 per cent transformation of the substrate. Bing (1964) with the same renin and the same sort of substrate preparation has shown that there is the same proportion in the case of a 20 per cent transformation of the substrate.

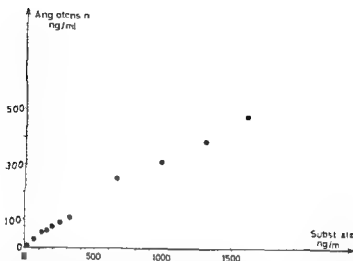


Fig 2

Shows the angiotensin produced at the varying concentrations of substrate. The concentration of substrate is expressed in ng of angiotensinogen measured as angiotensin. The concentration of renin (0.03 G U/ml) and the time (10 min) are kept constant.

Measurement of Known Renin Concentrations

The method includes 3 assays of angiotensin as described in 'Methods'. The *a* value is an expression of the sum of the concentrations of angiotensinogen from the unknown sample and the substrate at the start of the incubation.

The *b*-value is an expression of the remaining concentration of angiotensinogen after incubation for a number of hours, plus the *c*-value. The *c* value is the concentration of angiotensin after incubation for a number of hours, i.e. the part of the angiotensin produced that has not been digested by the angiotensinase.

The difference *b-c* is, consequently, an expression of the concentration of the angiotensinogen remaining after incubation for a number of hours.

The concentration of substrate is 1600 ng of angiotensinogen measured as angiotensin per ml at the start of the reaction. From Fig 2 it is seen that at a constant concentration of renin the angiotensin produced is proportional to the concentration of substrate at any time. When the angiotensin produced corresponds to the reduction of the concentration of the substrate, the concentration of renin in the reaction can be calculated by 1 order kinetics¹.

¹ From Fig 2 the formation of angiotensin is seen to be proportional to the concentration of the substrate (*S*). For these low concentrations of renin further more the angiotensin produced is proportional to the concentration of the enzyme (*E*). Thus the reduction of the concentration of the substrate per time unit $-\frac{dS}{dt} = k S E$. Integrating over times from $t=0$ to $t=1$, when *E* is kept constant we get equation (1) (Moore 1964).

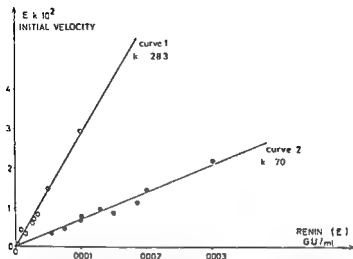


Fig 3

Indicates the initial velocity of the enzyme reaction at low concentrations of renin. Curve 1 and 2 have been attained with two different substrate pools, prepared in the same way and with the same concentration of angiotensinogen (This is seen in Fig 1). Marks as in Fig 1. The ordinate is proportional to the initial velocity since the concentration of substrate at the start of the reaction is kept constant.

$$\text{as } \frac{2303 \log \left(\frac{a}{b-c} \right)}{t} = E k \quad (1)$$

The left side of the equation can be calculated, t being the time in hours. The number 2303 is the conversion factor from natural to common logarithm. By this $E k$ can be calculated, E being the concentration of renin and k a constant.

In order to determine k , as mentioned in "Methods" a series of renin solutions with varying concentrations, corresponding to the physiological concentrations, is produced and $E k$ calculated. If $E k$ is plotted against the known concentrations of renin (E), the constant (k) is determined as the slope of the curve (Fig 3, curve 1).

The straight line found is assumed to indicate that, within the exactitude of the assay, the reaction is in accordance with 1 order kinetics. The straight line is produced with the incubation time varying from 19 to 48 hours, independently of the concentrations of renin. This shows that renin is stable during the whole period of incubation. Furthermore, the straight line is seen to pass through the zero point of the co-ordinate system, which corresponds to the fact that the whole reduction of the substrate concentration is exclusively due to renin.

Measurement of Renin in Plasma

When a standard curve (curve 1 Fig 3) is available, the concentration of renin in plasma can be determined as described in "methods".

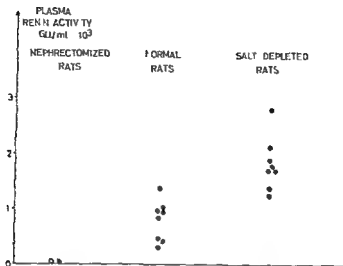


Fig 4

The renin activity in plasma is measured in 8 normal and 8 saltdepleted rats using double tests. No renin activity is found in two plasma pools from nephrectomized rats (see Fig 1)

by calculating E_k according to equation (1) as described above. Inhibitors or accelerators of the renin-angiotensinogen system in the plasma in question are able to modify the result.

The method is applied to 8 normal rats and 8 rats which have been saltdepleted through feeding from 4 to 28 days with potatoes and fresh distilled water. The results are seen in Fig 4.

Factors Modifying the Velocity of the Renin Angiotensinogen Reaction

If the substrate used in Fig 3 curve 1 is incubated with the concentration of renin increased to 0.025 and 0.030 GU per ml, the proportionality is seen to continue (Fig 5) since the slope of the curve is unchanged. The formation of angiotensin is measured direct by 10 minutes' incubation. In this period, as mentioned above, no demonstrable angiotensinase activity is found. In the determination of the velocity of the reaction the total angiotensinogen concentration at the start of the reaction replaces the a -value, and the difference between the total angiotensinogen concentration at the start of the reaction and the concentration of angiotensin produced by 10 minutes' incubation replaces the quantity $b-c$ in equation (1).

For another substrate pool, given exactly the same treatment as the substrate pool used in Fig 3 curve 1, we get another velocity of the reaction when low concentrations of renin are applied (Fig 3, curve 2). For higher concentrations of renin we get the same velocity of the reaction in the two substrate pools (Fig 5). This seems to indicate that in the substrate used for curve 2, Fig 3 there is an inhibitor which makes itself felt only in low concentrations of renin.

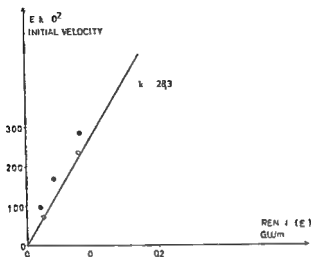


Fig 5

Indicates the initial velocity of the enzyme reaction at higher concentrations of renin. The two curves have now within the exactitude of the determinations the same slope corresponding to that of curve 1 Fig 3. Marks as in Fig 3. The determination for 0.08 GU/ml is the average of 8 determinations for each pool. Comparison with Fig 3 indicates the presence of a renin inhibitor only of consequence to low concentrations of renin.

DISCUSSION

It is desirable to measure renin under conditions similar to those in whole plasma, without inactivating angiotensinase. In the method described this demand is partly satisfied, the activity of angiotensinase being only slightly inactivated by a transitory acidification, which renin is known to tolerate without loss of enzymatic activity (Haas *et al* 1963).

Total inactivation of angiotensinase activity is made unnecessary since the principle of the analysis is determination of the reduction of the substrate concentration instead of measurement of the angiotensin produced. The determination of the concentration of the substrate is easily made in a plasma with an angiotensinase activity so high that the determination of the physiological concentrations of renin in plasma by measuring the angiotensin produced would be impossible.

In addition to the angiotensinase activity the concentration of the substrate is controlled during the incubation since the variation in the concentration of angiotensinogen in the plasma in question cannot influence the result because this variable is included in the determination of the reduction of the substrate concentration. The velocity of the reaction would then be expected to be proportional to the concentration of renin in plasma.

However the difference between curve 1 and Fig 3 seems to indicate the presence of an unknown factor in plasma from nephrecto-

mized rats (and probably also in that of normal rats) which alters the velocity of the renin-angiotensinogen system

This unknown factor is different from renin, angiotensinogen, and angiotensinase, and is measured independently of the increase in the concentration of angiotensinogen developed after nephrectomy (Bing 1964) For high renin concentrations no difference is seen between the two plasma pools, but for low renin concentrations the velocity is decreased in one of the plasma pools This was to be expected for a renin inhibitor Boucher *et al* (1964) and Pickens *et al* (1965) have observed that the formation of angiotensin is more rapid in some human plasma than in others

By determination of the renin activity in plasma from normal and saltdepleted rats with the method described, the renin activity is found to be highest for the saltdepleted animals This is in accordance with the results obtained by Brown *et al* (1963) and Genest *et al* (1966) for human plasma and by Fasciolo *et al* (1964) for dog plasma

SUMMARY

A simple microtechnique for quantitative measurement of renin in plasma is described For each determination 300 μ l of plasma is used The lowest concentration of renin measured is 0.0001 Goldblatt Dog Unit per ml, but the sensitivity limit can be increased by increasing the time of incubation The renin activity is found to be higher in saltdepleted than in normal rats, while no activity is found in 24 hours nephrectomized rats Variability in the velocity of the reaction found in two pools of plasma from nephrectomized rats may be explained by the presence of an unknown factor, probably a renin inhibitor, in the plasma

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By determination of the renin activity in plasma from normal and salt-depleted rats with the method described, the renin activity is found to be highest for the salt-depleted animals. This is in accordance with the results obtained by Brown *et al* (1963) and Genest *et al* (1966) for human plasma and by Fasciolo *et al* (1964) for dog plasma.

SUMMARY

A simple microtechnique for quantitative measurement of renin in plasma is described. For each determination 300 μ l of plasma is used. The lowest concentration of renin measured is 0.0001 Goldblatt Dog Unit per ml, but the sensitivity limit can be increased by increasing the time of incubation. The renin activity is found to be higher in salt-depleted than in normal rats, while no activity is found in 24 hours nephrectomized rats. Variability in the velocity of the reaction found in two pools of plasma from nephrectomized rats may be explained by the presence of an unknown factor, probably a renin inhibitor, in the plasma.

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aminocaproic acid (EACA) It should therefore be possible with such a therapy after trauma to accentuate the coagulation disturbance and thus increase the tendency to thrombus formation

The purpose of this study was after intravenous injection of tumour cells to investigate in isologous tumour host systems I The effect of trauma on the number, average volume and distribution of metastases in two different systems II In one of the systems to investigate the effect of heparin and EACA and also the combinations of trauma and EACA on the same factors

MATERIAL AND METHODS

The experimental studies have been performed on inbred C3H mice with a spontaneous mammary cancer and on CBA mice with a 20 methylcholantrene induced rhabdomyosarcoma MCG1 (Vellgren *et al* 1966)

A The C3H mice originated from Jax Vice Laboratory USA 1962 The mammary cancer developed in a female mouse 1963 and has thereafter been transplanted intramuscularly This study was performed on the 22nd generation Spontaneous

were counted in a hemacytometer and the viability of the cells was checked by
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were

- | | |
|-----------|--|
| Group I | 20 mice given 0.1 ml saline intravenously 3 hours before the tumour cell injection |
| Group II | 20 mice given 2 mg heparin (Vitrum Stockholm) in 0.1 ml solution subcutaneously two hours before and 3 hours after the tumour cell injection |
| Group III | 20 mice given 30 mg EACA (Kabi Stockholm) in 0.1 ml solution intravenously two hours before and 1 and 4 hours after the tumour cells injection |
| Group IV | 20 mice given 0.1 ml saline intravenously 2 hours before the tumour cell injection One hour before the cell injection both thighs were crushed with pliers under ether anesthesia fracturing the bone and crushing the muscles |
| Group V | 20 mice treated as in Group III and also subjected to crush fractures as in Group IV |

The effect of heparin on the coagulation time and of EACA on the inhibition of fibrinolytic activity were tested as described previously (Boeryd 1965) The coagulation time was not below 15 minutes during 1. hours after the first heparin injection The time for lysis of the coagulum in the EACA treated mice did not fall below 10 minutes if two hours before and 75 hours after the

the BSS were injected in a tail vein The mice in Group II and 11 mice in group IV died

B 30 CBA mice were divided into two groups

- | | |
|----------|---|
| Group I | 10 mice without treatment |
| Group II | 20 mice subjected to bilateral crush fracture of the thighs one hour before the tumour cell injection |

The tumour cell suspension of MCG1 was prepared according to Madden & Burk (1961) with some modifications by Boeryd *et al* (1965). Cell viability was tested with Trypan blue and was in the suspension used 96 per cent. The cells were counted in a haemocytometer.

50 000 cells in 0.1 ml modified Parker 199 (Sall *et al* 1954) were injected in a tail vein. The observation period was 18 days. 3 mice in group II died during this period and were discarded.

In both experiments the lungs and livers were examined microscopically. The number, average and total volumes of metastases were estimated as described previously (Boeryd *et al* 1966). Differences between groups were tested by Wilcoxon's two sample rank test (Hjgrenius 1962). Difference with $P < 0.05$ were accepted as significant.

RESULTS

A C3H mice At autopsy gross metastases were found only in the lungs. Microscopical examination of the lungs and livers disclosed metastases only in the lungs of all animals. The results are given in Table 1.

TABLE 1
Metastases to Lungs in C3H Mice

Groups		Incidence of takes	N	$\bar{v} \cdot 10^6$	$V \cdot 10^3$
I	Controls	20/20	2291	11	24
II	Heparin	18/18	3003	9	28
III	EACA	20/20	4868	17	84
IV	Trauma	9/9	4792	8	39
V	Trauma + EACA	20/20	9264	14	134

Heparin treatment increased the number of metastases to the lungs and reduced their average volume, but their total volume was unchanged compared to the controls.

EACA treatment increased the number, and average and total volumes of metastases to the lungs compared to the controls.

Trauma increased the number and total volume of metastases but left the average volume unchanged compared to the controls.

When trauma was combined with EACA the number as well as the total volume of metastases in the lungs were increased, but the average volume was not changed compared to the controls.

When the EACA-treated group was compared with the traumatized EACA-treated animals, the number of metastases and their total volume were found to have increased in the latter group, the average metastasis volume tended to be reduced ($0.1 > P > 0.05$).

When the traumatized mice were compared to the traumatized EACA-treated mice, an increase of the number and total volume of metastases was evident in the latter group but there was only a tendency to increased average volume ($0.1 > P > 0.05$).

B CBA mice At autopsy gross metastases were found in the liver

and in 4 of 10 controls and 6 of 17 traumatized animals in subcutaneous tissue kidneys mesentery diaphragm pelvic fat tissue and lymph nodes. The gross metastases did not differ significantly between the two groups. Microscopical examination disclosed that 8 of 10 controls and all traumatized animals had metastases to the lungs all controls and 15 of 17 traumatized animals to the liver. The results are given in Table 2.

TABLE 2
Metastases to Lungs and Liver in CB4 Mice

	Incidence of takes	N	$\bar{v} \cdot 10^{-6}$	$\bar{V} \cdot 10^3$
To lungs				
Controls	9/10	127	16	2
Trauma	17/17	216	20	26
To liver				
Controls	10/10	116	840	97
Trauma	15/17	28	748	43

Trauma increased the number, average volume and total volume of metastases to the lungs but reduced the number of metastases to the liver. The average volume of metastases to the liver was unchanged but the total volume tended to diminish ($0.1 > P > 0.05$).

DISCUSSION

Previous studies on the effects of heparin and EACA on the metastasizability of intravenously injected MCG1 tumour cells (Boery 1966) demonstrated that thrombus formation around the tumour cells was neither necessary for nor seemed to promote the establishment of metastases in that tumour host system. For prolonged heparin treatment merely redistributed the metastases from the lungs to the liver although the hepatic metastases were larger than in the controls.

In the present investigation the number and average and total volumes of pulmonary metastases from intravenously injected MCG1 tumour cells were increased after trauma whilst the number of metastases to the liver was reduced. Obviously this must have been due to tumour cell retention in the lungs. Trauma is attended by both increased tendency to thrombus formation and disturbed microcirculation. But as thrombus formation around the tumour cells seems not to promote metastasizability in this system the disturbed microcirculation may have been the decisive factor modifying metastasis development in the lungs. Tumour cell retention could also be due to increased aggregation of tumour cells which may lead to increased average tumour volume.

The C3H mice had no gross extrapulmonary metastases but this does not exclude the presence of minute such metastases elsewhere.

Heparin treatment was in these animals followed by an increase of the number of metastases to the lungs a result opposite to that observed by other authors using homologous systems (Cliffon & Agostino 1962 1963 Wood 1961 1964) but agreeing with results obtained by Boeryd (1966) using an isologous ascites tumour in CBA mice. The mammary cancer in C3H mice and the ascites tumour are similar in that they produced metastases almost solely to the lungs after intravenous tumour cell injection. The increased number of metastases after heparin treatment could be due to heparin facilitating transcapillary passage and repeated recirculation of tumour cells (cf Koile 1964) in time causing a higher number of cells to be trapped in the lungs. This in turn could be due to that heparin reduces trapping of tumour cells in thrombi. The results also could be due to that heparin reduces aggregation of tumour cells. In this tumour host system thrombus formation around tumour cells is not a pre requisite for the establishment of a metastasis.

The effect of EACA on the formation of pulmonary metastases may be due to increased retention of tumour cells in the lung. The increased average volume may be due to increased aggregation of tumour cells or/and inhibition of the spontaneous fibrinolysis exerted by FAC. The formation of microthrombi around trapped tumour cells could thus promote the growth of metastases. This promoting effect of fibrin may be due to fibrin being a suitable substrate for the growth of tumour cells (O'Veara 1958).

The effect of trauma on the number of pulmonary metastases may also be due to increased retention of tumour cells which could result from increased adhesion of tumour cells to the vascular endothelium possibly because of increased thrombus formation or disturbed microcirculation. Trauma had however no effect on the average metastasis volume. During the first 24 hours after trauma fibrinolytic activity of rat blood is increased (Rudenstam & Nilsson) and this could decrease the size of any thrombi formed around tumour cells. A diminished fibrin layer around trapped tumour cells may as suggested also interfere with the nourishment and growth of tumour cells and account for the non increased average metastasis volume.

When traumatized animals were treated with EACA in order to inhibit the increased fibrinolytic activity thus further increasing the tendency to thrombus formation the number of metastases to the lungs increased markedly. The reason for this is probably a more pronounced retention of tumour cells for the same reasons as after only trauma and after only EACA treatment. The average volume was unchanged compared with controls, tended to increase compared with the traumatized animals (Group IV) but tended to be reduced compared with EACA treated mice (Group III). This change of the average

metastasis volume in comparison with traumatized animals supports the hypothesis that thrombi around tumour cells may promote metastasis growth in this tumour-host system. Heparin treatment however, disclosed that such thrombus formation is not a pre requisite for metastasis growth.

SUMMARY

The effect of trauma on metastasis formation from intravenously injected tumour cells has been studied in two isologous systems. In one of these systems the effects of heparin, EACA and trauma + EACA have been studied too.

In one system, CBA mice inoculated with MCG1, trauma increased the number, average and total volumes of metastases to the lungs and decreased the number to the liver.

In the other system, C3H mice inoculated with mammary cancer, in which only pulmonary metastases developed, trauma increased the number and total volume of metastases, while EACA increased the number, average and total volumes compared to the controls. EACA treatment of traumatized animals led to a further increase of the number and total volume of metastases compared to the controls. In the latter system, however, heparin treatment also increased the number of metastases to the lungs.

These somewhat different effects of trauma in the two systems and the interplay between effects of heparin, EACA, trauma, and trauma + EACA have been discussed.

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Ph¹ PREVALENCE, PERIPHERAL BLOOD PICTURE AND CYTOSTATIC THERAPY

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Tough et al in 1962 reported that the decreasing leukocyte count in the peripheral blood during radiation treatment of the spleen in patients with chronic myelogenous leukaemia (CML) is accompanied by decreasing Ph¹ prevalence in the corresponding blood cultures. Other investigations have since demonstrated that comparable conditions occur during treatment with cytostatics (*Fitzgerald et al* 1963, *Carbone et al* 1963, *Whang et al* 1963). These results, together with demonstration of correlation between frequency of immature myeloid cells in the peripheral blood and Ph¹ prevalence in the corresponding blood cultures (*Fitzgerald et al* 1963, *Wolf et al* 1966), suggest that the Ph¹-positive mitoses in the blood cultures originate from the myeloid cell series. Whereas the Ph¹ prevalence in the blood cultures from patient to patient and at different times in the same patient vary just as much as the prevalence of immature myeloid cells in the blood, the frequency of Ph¹ positive cells in uncultured bone marrow is found, in practically all cases, to be 100 per cent or nearly 100 per cent, irrespective of antileukaemic treatment (*Sandberg et al* 1962, *Fitzgerald et al* 1963, *Tough et al* 1963, *Carbone et al* 1963, *Whang et al* 1963, *Tjio et al* 1966).

These investigations deal with essential conditions for the question whether cytogenetic observations can contribute towards illustration of the mechanisms concerned in progression of chronic myelogenous leukaemia. The present work was undertaken with the object of supplementing them with further investigations of the Ph¹ prevalences in the blood during cytostatic therapy and of the relationship between the frequencies of immature cells in peripheral blood and Ph¹ positive cells in blood and bone marrow.

MATERIAL AND METHODS

The cytogenetic materials or from 30 patients with Ph¹ positive chronic myelogenous leukaemia (CML) were obtained from blood cultures and in all of

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the patients. The extent of the blood culture material, the procedure employed in collecting the specimens and of blood culture and preparation of the specimens from the cultured materials is mentioned elsewhere (Pedersen 1966 a) where similarly, the principles for selection of the mitoses for cytogenetic analysis and the procedure employed in this analysis are described. All of the cultures in the present material were grown with phytohaemagglutinin.

The relationship between cytostatic therapy and Ph^+ prevalence in the blood culture was investigated in nine patients who were newly diagnosed and still untreated at the first investigation and in three patients who had been submitted to treatment for 8, 14 and 18 days, respectively, at the first investigation. The untreated patients were re-examined 41-222 days (average 111 days) later while the three patients who had undergone brief therapy were re-examined 44, 52 and 100 days (average 53 days) later.

The blood samples from which the cell cultures in the material originated were allowed to sediment spontaneously prior to incubation. The author undertook differential counting in the supernatant fluid prior to culture corresponding to 45 blood cultures from 23 patients. In each case the differential count comprised 400 cells. These counts were correlated with the Ph^+ prevalence of the corresponding cultures in order to illustrate further the relationship between the Ph^+ positive and immature myeloid cells.

The principles for collecting bone marrow specimens are mentioned elsewhere (Pedersen 1966 b). Eighteen bone marrow specimens comprising 550 cells were prepared without preliminary culture according to a modification of Tjio & Hwang's method (1962). Spreading of the mitoses was undertaken by evaporation of the fixative during air drying. In 13 bone marrow specimens comprising 322 cells the fixative was replaced by 60 per cent acetic acid. Spreading of the mitoses was subsequently undertaken by evaporation of the acetic acid.

The Ph^+ prevalences were calculated in the following manner: $(\text{No } Ph^+ \text{ positive cells} \times 100) / (\text{No } Ph^+ \text{ positive} + \text{No } Ph^+ \text{ negative cells})$. All cells lacking one or more small acrocentric chromosomes and where Ph^+ could not be identified were classified as $Ph^?$ cells and were therefore excluded from the formula recorded above.

RESULTS

The relationship between cytostatic treatment and Ph^+ prevalence in the blood cultures was investigated by comparison of the frequency of Ph^+ -positive cells in nine patients before and after periods of treatment of varying length (Table 1). A corresponding comparison was undertaken in three patients who had been treated with cytostatics for 14, 6 and 18 days, respectively, at the time of the first investigation and for 58, 68 and 69 days, respectively, at the time of the second investigation (Table 2). In 11 out of the 12 patients the Ph^+ prevalence was significantly lower at the second investigation. In one patient (Case 9) the leukocyte count was not available and in another case (Case 4) the differential count at the second investigation was missing. In the remaining nine patients, the haematological conditions had improved considerably during the period in which the fall in the Ph^+ prevalence had developed. Further, Case 9 showed a considerably improved differential count and Case 4 a restoration of the leukocyte count. In one patient (Case 8) the frequency of Ph^+ -positive cells had certainly diminished but not significantly. Corresponding to this the leukocyte count and blood picture were practically unchanged.

This parallel development between Ph^+ prevalence and the frequency of immature myeloid cells suggests that Ph^+ -positive and myeloid cells

TABLE 1

Haematological Data and Ph λ Distribution in Corresponding Blood Cultures Before and After Various Periods of Cytostatic Treatment

Case no	Date of sampling	Days in treatment	Ph λ positive cells	Ph λ negative cells	P	Leucocyte count ($\times 10^3$)	ht(%) ^a	Differential pmy(%) ^b	Count my(%) ^c †
1	23.1.63	-	26	7	< 0.0005	332.0	61	2	3
	30.2.63	27	-	47		0.9	8	2	-
	1.7.63	-	43	9	< 0.0005	251.0	4	-	26
	14.8.63	19	13	31		75.4	3	-	13
3	21.8.63	-	23	19	< 0.0005	161.6	9	-	20
	28.11.63	55	1	43		5.3		-	9
4	29.9.63	-	11	18	< 0.0005	190.0	22	-	22
	14.11.63	52	-	47		18.0			
5	4.10.63	-	18	29	0.0005 0.0010	71.1	3	8	12
	2.12.63	33	2	43		5.8	-	-	-
	15.11.63	-	41	7	0.0005 0.0010	255.2	1	6	23
	20.1.64	66	24	23		86.6	-	7	10
	9.12.63	-	23	21	< 0.0005	83.2	-	4	18
	23.3.64	81	-	27		6.4	-	-	1
8	5.2.64	-	8	21	0.05 0.10	160.0	-	13	23
	4.6.64	114	13	88		176.0	4	-	28
9	23.3.64	-	9	39	0.001 0.005	190.4	1	16	30
	31.10.64	17	-	50		-	-	-	8

misc. blasts

^a jr myelocytes

^c myelocytes

TABLE 2
Hematologic Data and Ph ϕ Distribution in Corresponding Blood Cultures after Different Periods of Cytostatic Treatment

Case no	Date of sampling	Days in treatment	Ph ϕ positive cells	Ph ϕ negative cells	P	Leucocyte count ($\times 10^3$)	bl(%) [*]	Differential pmy(%) [§]	Count my(%) [†]
10	13 7 63	14	46	2	< 0.0005	104.0	4	4	19
	26 8 63	58	21	22		45.6	-	-	16
	29 11 63	6	37	43		205.0	-	3	14
11	20 1 64	68	2	12	< 0.0005	11.0	-	-	3
	11 1 64	17	26	26		186.0	-	10	24
12	3 3 64	69	21		0.005	36.2	-	4	9

* See Table 1

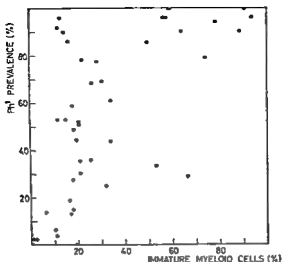


Fig 1

Percentages of immature myeloid cells in 45 leukocyte suspensions and Ph1 frequencies in blood cultures established from the leukocyte suspensions

are identical. An attempt has been made to illustrate this question further in Fig 1 where the results of the differential counts are compared with the Ph¹ prevalences in the corresponding blood cultures. The differential counts were carried out on the cell suspensions from which the blood cultures were established. Fig 1 shows the frequency of immature myeloid cells (myeloblasts, promyelocytes and myelocytes) in 45 cell suspensions from 23 patients. Suspension investigations were not carried out in four patients. Two patients showed peculiar conditions and are, therefore, not included in Fig 1 but will be dealt with separately later.

It is apparent from Fig 1 that there is a certain connection between the proportions of immature myeloid cells in the suspensions and the proportions of Ph¹-positive cells in the corresponding blood cultures. The points in the diagram, however, show great scatter. Possible causes of this scatter will be discussed later. The relationship between the prevalences of immature and Ph¹-positive cells describes an S-shaped curve. The proportions of Ph¹-positive cells show variations particularly in suspensions with 10-30 per cent immature myeloid cells. As it must be presumed that lymphocytes are the precursors of the Ph¹ negative cells in the cultures (Carstairs 1962, MacKinney *et al* 1962, Porter & Cooper 1962, Illies & Wilkinson 1963), and as the lymphocyte prevalences of the suspensions are not taken into consideration in drawing the diagram, it may be concluded that varying lymphocyte counts are responsible for the scatter of the Ph¹ prevalences. In 23 cultures, 10-30 per cent of immature cells were present. In Table 3, these cultures are subdivided into three groups according to the Ph¹ prevalences. As is

apparent, the lymphocyte content of the suspensions diminishes with increasing Ph^1 frequency

Table 3 suggests that the proportion of Ph^1 positive cells in the blood cultures is determined by the balance between the content of lymphocytes and granulocyte precursors in the initial leukocyte suspension. In order to illustrate this relationship in more detail the cultures from Fig. 1 were subdivided in Table 4 according to the content of lymphocytes and granulocyte precursors in the initial leukocyte suspension. As is apparent from the Table leukocyte suspensions with more or less uniform proportions of lymphocytes show higher Ph^1 prevalences; the higher their content of granulocyte precursors. Suspensions with more or less uniform content of immature myeloid cells appear to result in Ph^1 prevalence inversely proportional to the initial proportion of lymphocytes.

TABLE 3

Average Lymphocyte Percentages in Blood Cultures with Different Ph^1 Frequencies and 10-30 Per cent Myeloid Cells

Ph^1 frequency (%)	No. of cultures	Average lymphocyte frequency (%)
0-40	9	6.4
41-60	7	4.4
61+	7	2.1

TABLE 4

Frequencies of Immature Myeloid Cells and Lymphocytes in 45 Leukocyte Suspensions and Ph^1 Prevalences in Corresponding Blood Cultures

Lymphocytes (%)		Immature myeloid cells (%)			Total
		0-19	20-29	> 29	
0-4	Cultures	7	4	11	22
	Ph^1 positive cells	155	101	361	617
	Ph^1 negative cells	161	74	77	312
	Ph^1 prevalence (%)	49.1	57.7	82.4	66.4
> 4	Cultures	13	8	5	26
	Ph^1 positive cells	153	116	115	384
	Ph^1 negative cells	462	120	104	686
	Ph^1 prevalence (%)	24.9	49.2	52.5	35.9

As mentioned previously peculiar relationships were found between the content of granulocyte precursors in the blood and the content of Ph^1 positive cells in two patients. One of the patients (Table 5) a male aged 43 years was in a state of clinical and hematological exacerbation during the period of investigation from the time of the second investigation on 12/10/1962. Despite intensive treatment with cytostatics and corticosteroids no noteworthy improvement took place in his

TABLE 5
Peripheral Blood Picture and Ph1 Distribution in Blood Cultures and Direct Bone Marrow Specimens from a Patient in Acute Exacerbation of Chronic Myelogenous Leukemia

Date of sampling	Tissue	Ph1-positive cells	Ph1-negative cells	Ph1 prevalence (%)	Leucocyte count ($\times 10^3$)	bl ^a	Differential count (%) pmy ^a	my ^a
14 8 62	marrow	36	—	100.0	45.0	—	1	31
12 10 62	marrow	16	—	100.0				
	blood culture	4	38	9.5	62.4	48	5	3
15 10 62	blood culture	1	12	7.7	16.0	28		8
17 10 62	blood culture	1	48	2.0	20.4			
17 1 63	blood culture	4	41	8.9	16.2	36	—	2
21 1 63	blood culture	—	21	—	168.0	90	—	2
5 2 63	blood culture	—	20	—	201.0	98	—	—
11 2 63	blood culture	1	32	3.3	248.0	90	—	—

^a Table 1

TABLE 6
Blood Picture and Ph1 Distribution in Blood Cultures and Direct Bone Marrow Specimens from a Patient Before and After Acute Transformation of Chronic Myelogenous Leukemia

Date of sampling	Fluore	Ph1 positive cells	Ph1 negative cells	Ph1 prevalence(%)	Leucocyte count (x103)	hl* pmv* my*	Differential count (%)
23 9 63	marrow	13	-	100.0	190.0	22	22
14 11 63	blood culture	31	18	63.3	18.0		
16 4 64	marrow	21	47	100.0	20.4		
23 4 64	blood culture	6	49	10.9	13.0	65	5
24 6 64	blood culture	1	49	2.0	13.6	40	2
		5	99	4.8		33	1
							8
							3

* See Table 1

condition and this patient died on 12.2.1963 from bronchopneumonia. In connection with the two first investigations a bone marrow biopsy showed 100 per cent Ph^1 prevalence. Bone marrow investigations could not be carried out later on account of thrombopenia with a haemorrhagic tendency. Despite the immature blood picture the Ph^1 prevalences were always found to be under 10 per cent. The karyotypes of the Ph^1 positive cells examined did not show any abnormalities apart from sporadic hypodiploidy.

The second patient (Table 6) a female aged 46 years (identical with Case 4 in Table 1) was investigated for the first time prior to the commencement of treatment with cytostatics. On this and a later occasion Ph^1 frequencies in the bone marrow of 100 per cent were found. The first blood culture showed a high prevalence of Ph^1 positive cells. At the next investigation the patient was in a state of good remission. The content of Ph^1 positive cells in the blood culture was in good agreement with this *viz.* 0 per cent. At the subsequent investigations the condition was clinically and haematologically poor but the Ph^1 frequencies in blood culture were low. Scarcely half of the Ph^1 positive marrow and blood culture cells from April and June 1964 had the same aneuploid karyotype with 47 chromosomes, the (21/22) group containing two Ph^1 . This patient died from bilateral bronchopneumonia six weeks after the last cytogenetic investigation.

The prevalence of Ph^1 positive cells in uncultured bone marrow was investigated in 31 biopsy specimens from 24 patients and comprised a total of 872 analysed cells. Of these 792 were Ph^1 positive, 28 Ph^1 negative and 52 cells proved impossible to classify.

Twenty seven of the 28 Ph^1 negative bone marrow cells originated from one particular sample of bone marrow, the cells of which were distributed in the following manner: Ph^1 positive 6, Ph^1 negative 27 and $\text{Ph}^{1?}$ 1. The sample originated from a male aged 70 years whose illness commenced in 1961 with fatigue, night sweats, loss of weight and enlargement of the spleen. The bone marrow and the peripheral blood showed histological changes typical of the disease. Good remission was obtained with Busulfan. At the time of the investigation the leukocyte count was 80 000 and was increasing. The spleen could be palpated 10–12 cm below the left costal margin. Unfortunately no differential counts from the blood or bone marrow are available from this period. The patient did not exhibit any clinical or haematological signs of bone marrow insufficiency at any time. Culture of a blood sample withdrawn simultaneously with the marrow sample was undertaken but no growth resulted. As the patient died from cerebral haemorrhage (18.10.1962) a week after the cytogenetic investigation this was not repeated.

Where seven of the 10 marrow specimens were concerned no differential counts from the peripheral blood were available. The remaining 24 samples are included in Table 7 on

the basis of the frequency of immature myeloid cells in the blood. Regardless of the character of the peripheral blood picture, the prevalence of Ph¹-positive cells in the bone-marrow was 100 per cent or practically 100 per cent.

TABLE 7

Percentages of Immature Myeloid Cells in Peripheral Blood and Ph¹ Prevalences in 24 Direct Bone-Marrow Specimens

Blood picture Immature myeloid cells(%)	Samples	Ph ¹ positive cells		Ph ¹ negative cells		Ph ¹ ? cells number
		Number	prevalence(%)	Number	prevalence(%)	
0-19	3	134	100.0	-	-	6
20-39	12	365	99.7	1	0.3	24
> 39	7	154	100.0	-	-	4
Total	24	653	99.8	1	0.2	34

In 18 out of the 31 samples in the bone-marrow material, the mitoses were spread by evaporation of the fixative. This material comprises 550 cells of which 52 (9.5 per cent) were hypodiploid. In 13 samples, comprising 322 cells of which 163 were hypodiploid (50.6 per cent), the fixative had been replaced by 60 per cent acetic acid and spreading of the mitoses was undertaken by evaporation of the acetic acid. The proportion of hypodiploid cells was significantly higher in the latter group ($P < 0.0005$), probably because the high temperature necessary to evaporate the acetic acid resulted in rupture of the cell membranes. As the high hypodiploid frequency appears to have been artificially conditioned, the material treated with acetic acid was not included in the bone-marrow material which was published previously (Pedersen 1966b).

DISCUSSION

In 1962, *Tough et al.* described a parallel decrease in the leukocyte count and the Ph¹ prevalences in the corresponding blood cultures in six patients with CML during radiation of the spleen. Later investigations have demonstrated a corresponding development during treatment with cytostatics (*Fitzgerald et al.* 1963; *Whang et al.* 1963; *Speed & Lawler* 1964). *Fitzgerald et al.* (1963) found, in addition, positive correlation between the prevalences of immature myeloid cells in the peripheral blood and Ph¹-positive cells in the corresponding blood cultures. Similar observations were made by *Whang et al.* (1963) and *Tee et al.* (1964).

The results of the present investigation are in good agreement with the investigations mentioned. The relationship between the content of myeloid cells in the initial cell suspensions of the blood cultures and their final content of Ph¹-positive cells is dominated, however, by the

great scatter of the observations. In addition there does not appear to be proportionality between the two variables as the observations do not describe a straight line but rather an S shaped curve (Fig 1)

As emphasized by *Court Brown & Tough (1963)* numerous factors condition variations in the relationship between the content in the peripheral blood of myeloid cells and the content of Ph^1 mitoses in the corresponding cultures when harvested

The results of differential counting are associated with uncertainty on account of the relatively limited number of cells counted. In order to reduce this uncertainty to a reasonable extent each count in Fig 1 was based on 400 cells. On the other hand the Ph^1 prevalences in the majority of cultures are based upon 20 cells analysed and thus these values have not inconsiderable uncertainty

Separation of the red and white blood cells prior to establishing of the cultures was undertaken by spontaneous sedimentation of the erythrocytes without addition of phytohaemagglutinin. During this sedimentation the cellular composition of the leukocyte population may be altered. Comparison of differential counts on whole blood and the corresponding leukocyte suspensions does not suggest any systematic difference. In 23 differential counts on whole blood in the present material an average of 30.5 per cent immature myeloid cells was found while the corresponding leukocyte suspensions showed an average of 29.1 per cent. In occasional cases however considerable differences between the corresponding counts were observed. In order to avoid these sources of error the observations in Fig 1 were based upon counts of the leukocyte suspensions

Incubation periods of different lengths may influence the proportions of Ph^1 positive cells in the culture (*Nowell & Hungerford 1961*). An attempt was made to avoid this source of error by keeping the period of culture as constant as possible in length. All of the cultures were harvested after 46-51 hours of culture

Uncontrollable variations in the experimental conditions may be anticipated to influence the ability of the leukemic cells to grow *in vitro*. Further it is possible that the distribution on myeloblasts, promyelocytes and myelocytes and the occurrence of abnormal karyotypes in the leukemic cell population may influence the competition *in vitro* between the leukaemic and non leukaemic cells and thus the Ph^1 frequency in the cultures

The modifying factors mentioned thus appear to be able to explain the scatter of the points in Fig 1. The S shaped distribution of the points is presumable conditioned by variations in the lymphocyte frequencies in suspensions with 10-30 per cent immature myeloid cells (Table 3)

In two patients the Ph^1 prevalences in the blood cultures were found to be very low despite high frequencies of immature cells in the peripheral blood (Tables 5 and 6). Bone marrow investigations in both

of these patients showed 100 per cent prevalence of Ph¹-positive cells. The number of blood cultures excludes technical factors as the cause of the low prevalences in both patients. Special defects in the myeloid cells concerned appear to have rendered *in vitro* proliferation impossible. Tough *et al* (1961) described two similar cases. Although the clinical course was typical of CML, it is difficult to exclude the possibility that the cases described by Tough *et al* might belong to the rare group of Ph¹-negative patients as bone marrow investigations were not available.

In contrast to the varying Ph¹ prevalences in the peripheral blood, the majority of investigations show constant frequencies of Ph¹-positive cells in uncultured bone-marrow regardless of treatment and phase of the disease. Tough *et al* (1963) thus found only one Ph¹-negative out of 1343 bone-marrow mitoses investigated from 25 patients. However, two patients had already been excluded from this material with exclusively Ph¹-negative bone-marrow and three patients with partially Ph¹-negative bone marrow and clinical and hematological signs of bone-marrow insufficiency. The results of the present investigation are in good agreement with this as all of the Ph¹-negative cells with one exception originate from one patient. This patient, who had 18 per cent Ph¹-positive cells in the bone-marrow did not show any clinical or hematological signs of bone-marrow insufficiency at any time during the period of disease.

Following exclusion of cells with questionable Ph¹, the material published by Sandberg *et al* (1962), which originates from 14 clinically typical patients, contains 9.2 per cent Ph¹-negative mitoses. In 15 samples from 13 patients, Whang *et al* (1963) found 88-100 per cent on an average of 97.8 per cent Ph¹-positive cells. Carbone *et al* (1963) found 96-100 per cent Ph¹-positive cells in 22 patients. In 14 bone marrow samples from ten patients Fitzgerald *et al* (1963) demonstrated 24-100 per cent of Ph¹-positive cells, on an average of 73.6 per cent. Tjio *et al* (1966) in their material from 60 patients found that on average of 1.2 per cent of the cells investigated were Ph¹-negative.

These results are possibly only apparently controversial. In the four first mentioned results, differentiation was undertaken only between Ph¹-positive and non-positive cells. The latter group probably includes the mitoses which do not prove possible to classify as regards Ph¹. In calculation of the Ph¹ prevalences the Ph¹-positive cells are compared with the total number of cells investigated which implies that the Ph¹⁺ cells are included in the calculation as Ph¹-negative. The genuine frequency of Ph¹-positive cells i.e. the Ph¹-positive fraction of the cells whose content or absence of Ph¹ can be determined with certainty is therefore probably higher than the number stated. In addition, the authors who give the number of Ph¹⁺ cells do not define this category more exactly. Hypodiploid cells which lack 21-22 members should be considered as Ph¹⁺ cells as artificial factors may

have caused loss of Ph^1 in these cells. *Fitzgerald et al* (1963) found very low Ph^1 prevalences in two patients. These were apparently patients with both Ph^1 -positive and Ph^1 negative populations in the bone-marrow similar to the three described by *Tough et al* (1963), the patient mentioned in the present investigation and a case described by *Speed & Lawler* (1964). Following exclusion of these two patients, the Ph^1 prevalence becomes 88.3 per cent in the material presented by *Fitzgerald et al*. This material contains rather a high proportion of hypodiploid cells i.e. 21.8 per cent compared with 9.5 per cent in the fraction of the present material which was treated according to *Tjio & Whang's* method (1962), which may be due to artificial chromosome loss. Presuming this, it appears possible that the frequency of hypodiploid cells which lack (21-22) members and are included as Ph^1 -negative cells in the calculations of Ph^1 prevalences is quite high and can explain the discrepancy to the other materials published.

The investigations quoted thus suggest that the marrow population in the majority of patients with CML consists exclusively of Ph^1 positive cells. This finding forms the basis of the presumption that not only the myelopoietic but also the erythropoietic and megakaryocytic systems are Ph^1 -positive (*Sandberg et al* 1962, *Tough et al* 1963). In 1963, *Whang et al* succeeded in demonstrating that the erythrocyte precursors may be Ph^1 positive and that it is probable that they are in many cases. *Tough et al* (1963) demonstrated in one case parallelism between the thrombocyte count in the blood and the frequency of polyploid Ph^1 positive mitoses in corresponding uncultured bone marrow samples. This finding supports the hypothesis about Ph^1 in the megakaryocytic system. The existence of patients with both Ph^1 -positive and Ph^1 negative bone marrow populations may possibly indicate that Ph^1 may occur only in myeloid cells in certain cases (*Whang et al* 1963).

The persistence of 100 per cent Ph^1 prevalence despite intensive antileukaemic treatment suggests that the preleukaemic Ph^1 -negative population becomes totally suppressed by Ph^1 positive cells or is no longer capable of proliferation. As the dosage of the antileukaemic therapy is, as a rule, regulated according to the effect upon the peripheral blood picture, it seems obvious why the Ph^1 prevalence in the bone marrow does not reflect any therapeutic effect.

However, provided other factors are equal, when a Ph^1 positive bone-marrow population exists in an environment containing a cytostatic, the variants whose proliferation is least inhibited by the cytostatic are favoured. It is therefore possible that conventional antileukaemic therapy, which has no or only a slight effect upon the bone marrow population, will encourage proliferation and further development of resistant cell variants and consequently development of intractable myeloblastic crises.

SUMMARY

In a material of blood cultures originating from 29 patients with clinically and haematologically typical chronic myelogenous leukaemia, the following questions were illustrated. The effect of cytostatic therapy on the Ph¹ prevalence in blood culture and the relationship between the content of lymphocytes and immature myeloid cells in the initial leukocyte suspensions and the corresponding Ph¹ prevalences after culture *in vitro* for 46-51 hours. Finally, the frequency of Ph¹-positive cells was investigated in uncultured bone-marrow from 22 patients.

1 In 11 out of 12 newly-diagnosed patients of whom nine were untreated at the first investigation and three had received treatment for 6, 17 and 24 days, respectively, marked decrease in the Ph¹ prevalences of the cultures were observed after periods of treatment of varying lengths. Simultaneously, the peripheral blood pictures improved considerably. In one patient, the frequency of Ph¹-positive cells did not decrease significantly and the blood picture did not show any definite improvement (Tables 1 and 2).

2 Prior to incubation, differential counts were undertaken on 45 leukocyte suspensions from 23 patients for comparison with the Ph¹ frequencies in the corresponding blood cultures (Fig 1). The observations revealed a greater proportion of Ph¹-positive cells in the cultures harvested the greater the proportion of immature myeloid cells in the corresponding initial suspensions. The relationship between the two variables is, however, not linear but describes an approximately S-shaped curve. The shape of the curve appears to be caused by varying lymphocytic content in the suspensions. In two patients, low Ph¹ prevalences were found despite very immature peripheral blood pictures. In these two cases, special defects appear to have rendered proliferation of the myeloid cells *in vitro* difficult (Tables 5 and 6).

3 In one patient, only six out of 33 bone-marrow mitoses investigated were definitely Ph¹-positive. This patient did not differ clinically and haematologically from the other 21 patients from whom bone-marrow investigations are available. These investigations comprise a total of 787 cells which could be classified with certainty as regards the presence or absence of Ph¹. Of these 787 cells, only one was Ph¹-negative.

The results of the present investigations are in good agreement with previous investigations and together with these they suggest that the immature myeloid cells of the peripheral blood are identical with the Ph¹-positive cells of the blood cultures and that decreasing Ph¹ prevalences in the blood cultures during successful therapy are a reflection of the improvement of the peripheral blood picture.

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CYCLES OF HAIR GROWTH IN HAIRLESS MICE

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A moderate single surface application of a carcinogen to mouse skin gives rise to many tumours when applied during the resting stage of the hair cycle whereas few or no tumours are seen when the carcinogen is applied during the growth phase. This hair cycle effect has been extensively discussed by *Engelbreth Holm* and his school (see *Borum* 1960).

It has several times been claimed that the hair cycle effect is probably of no or of minor importance in hairless mice. *David* (1932) has shown however that hairless mice have rudimentary hair cycles even if these are not easily observed. In the study of carcinogenesis it is of importance to know the time of appearance of these cycles. We have therefore studied the more or less rudimentary hair cycles in our strain of hairless mice.

MATERIAL AND METHODS

Hairless mice of the strain *hr/hr* were used (For a more detailed description see *Iversen & Friensen* 1962). The animals were housed in plastic cages and fed a standard diet. Groups of 4 male and 4 female mice were killed by neck fracture at different time intervals starting at an age of fifteen days (see Graph 1). The skin of each animal was flayed off stretched on a piece of cardboard and fixed in 4 per cent neutral formaline. Specimens were taken from the central as well as from the dorsal side and in each case from the cranial, the middle and the caudal part of the body. The specimens were embedded in paraffin and cut and stained with eosin and haematoxylin. The microscopic staging of hair growth phases follows the conventional nomenclature (see for instance *Argyris* 1963).

RESULTS

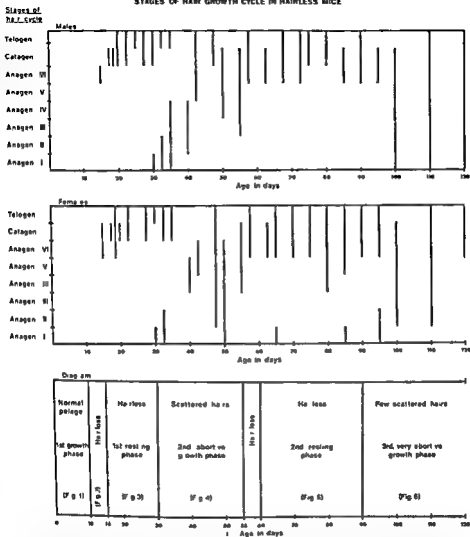
The results are summarised in Graph 1.

Hair Growth

Generally no difference between males and females was observed. The two sexes are therefore considered together.

At birth these mice are hairless but just afterwards they acquire a normal thick first pelage (Fig. 1) which they start losing again at the

STAGES OF HAIR GROWTH CYCLE IN HAIRLESS MICE



Graph 1

Stages of hair cycles in hairless mice. The vertical bars in the two upper parts represent stages of hair cycle observed in one or more of the animals studied at that age. The lower part of the graph is a diagram summarizing the findings, and with references to the photographs of mice at different ages.

age of 10 to 12 days. The hairs are always lost from the head tailwards (Fig 2), and the loss is complete after about ten days.

In the 13-day-old mice many hairs are still fully grown out, but the hair follicles show signs of degeneration (catagen stage, Fig 7). Some few hair bulbs are still well preserved, with normal hairs. Mitotic figures are not seen in the hair follicles at this time, and the inter-



Fig 1

A 1 week old mouse with apparently normal pelage

follicular epithelium is thin. Signs of degeneration of the hair bulbs are mostly seen in the specimens from the cranial part. Two to three days later, however, hair bulbs in the catagen stage are also observed in the remaining part of the skin area.

On the 20th day the animals are macroscopically hairless (Fig 3). Microscopically all hairs are in the catagen or in the telogen stage (Fig 8). This condition continues for about ten days.

About the 30th day almost all the animals reveal a number of mitotic figures in many hair follicles, and during the period from 30 to 40 days, the histological picture is characterized by many hair bulbs in different steps of the anagen stage.

At about the 40th day some slight regeneration of hair is macroscopically seen in most animals (Fig 4). This pelage, however, is far



Fig 2

A 13-day old mouse in the hair losing phase

from being so thick as the first one. In most of the mice only a few scattered hairs are observed but a small number of animals have a thin fur. Other animals again are almost hairless.

After the 20th day this second rudimentary pelage is lost and microscopically almost all follicles are again in the catagen or telogen stage. This second resting phase lasts from day 50 until the animals are about 90 days old (Fig 5). Some very few scattered follicles still show signs of hair growth even during this resting phase.

On the 90th day a third growth cycle starts but with only a few active follicles. One observes only a few hairs in some of the animals (Fig 6) but many remain hairless. Microscopically a few scattered follicles show signs of hair growth.



Fig 5

A 70-day old mouse with hairless skin



Fig 6

A 100-day old mouse with some hairs. This is an example of maximum hair growth at this age.

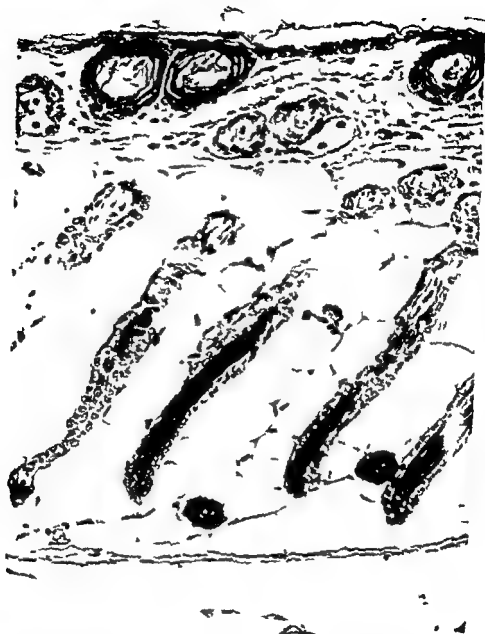


Fig 7

Microscopical section from the skin of a 15 day old mouse with hair bulbs partly well preserved partly with degenerative changes \times is the thickness of the skin Initial $\times=64$



Fig 8

Microscopical section from the skin of a 27 day old mouse Thin dermis without hairs Inactive hair bulbs Initial \—■

to the skin surface (Fig 10) These hairs may be surrounded by an inflammatory infiltrate, which also contains multinucleated foreign body giant cells These cysts and the inflammatory changes remain during the rest of the life of the animals

DISCUSSION

The strain of hairless mice *hr/hr* (sometimes called "Crew mice") is not absolutely devoid of hair Our strain is obtained from P Hertwig Biologisches Institut, Halle, Germany, in 1939, and has since been bred in our Institute The hairless mothers cannot take care of their offspring because their mammary glands are rudimentary In this laboratory females with hairs heterozygote for hairlessness, are mated to homozygote hairless males

Female offspring with hairs are used as mothers for the next generation, whereas hairless females are used for experiments The hairless males are used for experiments and for mating but strict brother-sister breeding cannot be performed Yet, with this type of breeding during 27 years this strain is if not purely inbred rather homogenetic

Our investigations show that there are at least two distinct growth cycles of the hair bulbs of these animals The first stage of growth



Fig 9

Microscopical section from the skin of a 43 day old mouse. Histiocytes and lymphocytes around active hair follicle. Cysts in the corium. Initial X = 61

lasts from birth, and the pelage is complete at the age of 10 days. The second growth phase starts about day 30, and lasts for about three weeks. A third, abortive growth phase starts in some animals 90 days of age.

As it is of importance to pay attention to the "hair cycle effect" in the study of carcinogenesis when using single application of carcinogens, such studies with hairless mice of the strain *hr/hr* should preferably start when the animals are in a sufficiently stable resting stage, i.e. between 60 and 90 days of age.

SUMMARY AND CONCLUSIONS

Hair growth cycles in the strain of hairless mice (*hr/hr*) are studied. Rather distinct phases of growth and rest are observed.

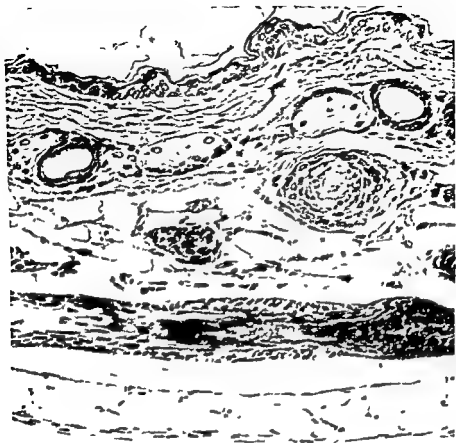


Fig 10

From the same mouse as Fig 9 A hair growing horizontally close to the musculus panniculus Initial $\lambda = 64$

The first growth phase lasts from birth until the mice are about 10 days old. This pelage is apparently normal. From the 12th to the 15th day these hairs are lost from the head tailwards.

The first resting phase lasts from the 15th to the 30th day. Almost no hairs are observed, and the hair bulbs are in the catagen or in the telogen stage.

The second growth phase lasts from the 30th to the 55th day. Some few scattered hairs are observed on the skin, and a few of the hair follicles are in the anagen stage.

The second resting phase lasts from about the 60th day to about the 90th day of age. During this period only very few scattered hairs are seen in some of the mice. Microscopically the hair bulbs are almost all in telogen or catagen.

After the second resting phase a very abortive third growth phase occurs in some few mice

Inflammatory changes are present from the time of the degeneration of the first pelage *i.e.* from the 20th day. Formations of cysts begin in the second growth phase

For single application of carcinogens during a resting phase mice at an age of 60 to 90 days should be used

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ON THE APPEARANCE OF THYROGLOBULIN AUTO-ANTIBODIES AFTER EXPOSURE OF NORMAL HUMAN THYROIDS TO SURGERY AND IONIZING RADIATION

By

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A variety of patients without thyroid disease may be exposed to thyroid injuries. Tracheostomy frequently requires splitting of the thyroid isthmus and malignant tumors of the neck may be treated by x rays striking the thyroid, or by surgery including intervention on the gland. Whether these injuries may initiate thyroid auto-immune reactions, appears to remain unknown. Investigations of such patients may both throw light upon this question and upon the mechanism by which thyroglobulin auto-antibodies reach detectable levels in human serum.

At present, possible mechanisms for the induction of thyroglobulin auto-antibodies may be summarized as follows:

1. The "leakage theory" (4, 13). By this hypothesis thyroglobulin is assumed to be secluded from the immune system in the tolerance developing period. If thyroglobulin later gets entry to the circulation, the molecule is recognized as "foreign" and stimulates antibody synthesis.
2. Disturbed tolerance theories. These hypotheses assume that tolerance towards thyroglobulin normally develops but may be terminated by either of the following mechanisms:
 - A. An alteration of the thyroglobulin molecule
 1. A structural change such as denaturation presenting "hidden" antigenic determinants (17).
 2. Addition of new antigenic determinants (28). Viruses may give rise to such new antigenic determinants of cells (26) or cellular products (23).
 - B. Secondary to thyroid disease not involving structural changes of thyroglobulin. Lymphoid cells may be attracted to the thyroid gland for other reasons than auto-immune reactions. By an infiltration of this kind, interactions between thyroglobulin and lymphoid cells different from the fate of thyroglobulin in lymph nodes may take place and favour auto-antibody synthesis.
 - C. Hyperactivity of the immune system. A disturbance located to the immune system "spontaneously" giving rise to so-called "forbidden clones" (3).
 - D. Molecules of the external milieu such as antigens of micro-organisms may have antigenic determinants closely related to tissue components and give rise to cross-reacting antibodies (1).

Since human thyroglobulin auto-antibodies frequently are found in a variety of thyroid disorders (24) many of which most unlikely have an auto immune etiology, the auto-antibody production seems to be secondary to the thyroid disorder. Furthermore, in many of these thyroid disorders the immune system reveals no sign of hyperactivity (9). On this basis, hypotheses 2 C and D may be excluded as main mechanisms behind thyroglobulin auto antibody formation.

Several observations do not support the "leakage" theory, as discussed by Hjort (13). One of the arguments against this theory is the lack of antibody response in patients after surgical treatment of thyroid disease, although surgical intervention on the thyroid gives rise to circulating thyroglobulin (10). Since circulating thyroglobulin frequently is found in serum of patients with thyroid disorders (11), the explanation could be that the selected group of patients without antibodies had become tolerant to thyroglobulin previous to surgery. Consequently, auto-antibody response after leakage of thyroglobulin should be studied in patients without thyroid disease.

The present report concerns thyroglobulin auto-antibody response in patients without clinical evidence of thyroid disease after injuries of their thyroid gland.

MATERIALS AND METHODS

The material consisted of 99 patients from the Ear, Nose and Throat Department at Rikshospitalet, Oslo. 86 patients suffered from tumors located to larynx or upper part of trachea. These patients were treated either by laryngectomy including hemithyroidectomy or by external x rays which strike the thyroid. The total x ray dose varied from 2000 R to 4000 R to one area on each side of the neck corresponding to a tissue dose in the thyroid of approximately 3400-6200 R. One patient suffered from a cerebral tumor and another from cervical spondylitis. Both were tracheostomized with splitting of the thyroid isthmus. The 11 last patients suffered from tumors of their larynx. These are included in the control group because serum specimens were only achieved prior to treatment.

TABLE 1
Time Interval between Thyroid Injury and Serum Sampling

Type of injury	Surgery	X rays
No. of patients tested \leq 2 months after injury	11	25
" 4-6 "	3	13
" 7-12 "	6	16
" 2-5 years "	10	28
" \geq 6 "	7	11

One or more serum samples were obtained from each patient at varying periods of time after treatment as shown in Table 1. Twenty five patients were tested both before and after treatment. The interval between treatment and antibody testing of these patients varied from 2 weeks to 6 months.

Thyroglobulin antibody was demonstrated by the indirect hemagglutination technique as previously described (6, 7, 8) employing formalized human erythrocytes containing approximately 35 000 antigen molecules per cell (8). The erythro-

cytes were used in a concentration of about 4×10^6 cells per ml counted by photometry (6). Sensitivity was controlled by a positive reference serum obtained from the Department of Bacteriology and Immunology, University of Buffalo School of Medicine, Buffalo, N.Y. (Head Professor F. Witelsky, M.D.). The sera were treated as previously described (7) and tested in dilutions of 1:1, 1:25, 1:250 and 1:2500 made by a single pipette. Sera with a positive titer = 1:2500 were tested further in ten fold dilutions for hemagglutination activity and undiluted in gel precipitation.

Precipitation in gel *ad modum Ouchterlony* (19) was performed as described by Eriksen (5) employing 0.03 M barbital buffer at pH 8.6, 0.003 per cent methyl orange, 1 per cent Difco Special Agar Noble and 0.1 per cent phenol.

RESULTS

The results are presented in Table 2. Out of 54 patients exposed to x rays only, 3 patients (5.6 per cent) revealed thyroglobulin auto antibodies in their serum. Two of these were tested 3 years after treatment. One patient, a 50 years old male, revealed a titer of 1:25 while the other, a 60 years old male, gave rise to a titer of 1:250. The third patient was a male of 74 years who both 6 weeks and 1 year after treatment showed an antibody titer of 1:25000 and a positive gel precipitation reaction. The last case is described in more detail below (Case 1).

TABLE 2
Age and Sex Distribution of Patients without Thyroid Disease Exposed to Different Types of Thyroid Injury

Age in years		21-30	31-40	41-50	51-60	61-70	71-80	81-90
Sex		♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀	♂ ♀
Type of injury	Total no tested							
X rays	54	1	1	4	16	10	8	2
No positive	3			1	1		1	
Surgery	14		1		5	6	1	1
No positive	1					1		
Surgery and x rays	7		1		3	2	1	
No positive	1					1		
X rays and surgery	13		1	1	3	6	1	
No positive	0							
Tested prior to injury	36		2	3	7	9	7	2
No positive	1					1		

Of 14 patients exposed only to surgery, 1 patient, a 67 years old male, operated 7 years prior to serum sampling, gave a positive reaction (1:25). Twenty patients were exposed both to x rays and surgery. In a single patient thyroglobulin antibodies were detectable. Serum obtained 6 weeks after a biopsy of the thyroid and 3 weeks



Fig 1

Thyroid tissue from patient FT. Arrow points to the infiltration of a squamous cell carcinoma surrounded by lymphocytes and fibrous tissue. In peripheral parts thyroid follicles with scattered lymphocytic aggregation may be seen (Hematoxylin and eosin $\times 90$)

after the beginning of x-ray treatment to the neck revealed a titer of 1:2500 and a negative gel precipitation reaction. This patient was tested 11 months prior to any treatment with a negative result and is described in further detail below (Case 2).

Altogether 5 out of 88 patients (5.7 per cent) with injured thyroids revealed thyroglobulin antibodies, while 1 out of 36 patients tested prior to any treatment gave a positive reaction. In 1 out of 25 patients a negative reaction previous to treatment was followed by a positive afterwards (Case 2).

Case 1. A male (J.B.) born 1891 was admitted to the hospital on September 4th 1964 for treatment of a right vocal chord carcinoma. No history of previous thyroid disease and no signs of struma or hypothyreosis by physical examination at admission.

From September 8th to October 20th he received a total amount of 3850 R to each side of his neck. Shortly afterwards he began to feel sensitivity to cold, dryness of the skin, tiredness, and he became constipated.

He was admitted for control of his laryngeal carcinoma on December 9th 1964. His tumor had then regressed. However, his face was swollen and he appeared suspect of myxedema. His thyroid was not palpable. Investigations: Protein Bound Iodine (PBI) 0.9 μg per ml, cholesterol 380 mg per 100 ml, ^{131}I uptake 4 per cent/4 hours and 3 per cent/24 hours. Thyroglobulin antibodies: hemagglutination titer 1:25,000, gel precipitation Positive.

Treatment with L-thyroxine 0.10 mg $\times 3$ was initiated and his symptoms of hypothyreosis disappeared gradually. Serum specimens from this patient obtained 4, 6, and 12 months after x-ray treatment gave identical results. A hemagglutination titer of 1:25,000 and a positive precipitation in gel.

Case III A male (ET) born 1900 was admitted to the hospital on May 10th 1965 because of hoarseness for the last few months. Four months earlier he obtained an x ray examination because of dysphagia and a Zenker's diverticulum was diagnosed. He was found to have a left sided paralysis of the recurrent nerve. His thyroid was not palpable. Bronchoscopic and oesophagoscopic examinations did not reveal any pathological changes except for the diverticulum of hypopharynx.

He was discharged from the hospital and controlled one month later and on October 4. The first time he was without symptoms except for the persistence of the recurrent paralysis but the last time he complained about cough and respiratory distress. At that time and in addition to the recurrent paralysis a supraclavicular lymph node on the left side suspect of metastasis was observed. For this reason he was readmitted to the hospital.

The respiratory distress was by bronchoscopy found to be caused by a tumor in the upper part of the trachea. Tracheostomy was performed on October 12. Tumor infiltration was found on the tracheal wall and in the upper part of the thyroid. Histology showed a squamous cell carcinoma originating in the trachea. The stroma around the tumor revealed a dense inflammatory reaction with infiltration of lymphoid cells and granulocytes. Also the metastasis to the thyroid apparently had attracted lymphoid cells as shown in a picture of the thyroid (Fig. 1).

On October 20 x ray treatment of an 8 x 10 cm area below the jugulum was

May 14 1965	Indirect hemagglutination negative
November 29 1965	Indirect hemagglutination 1:200 Precipitation in gel negative
March 16 1966	Indirect hemagglutination 1:200 Precipitation in gel negative

DISCUSSION

Thyroglobulin auto antibodies were found in the serum of 3 patients out of 54 (approximately 5.6 per cent) without clinical signs of thyroid disease exposed to x rays of their thyroid in 2 out of 34 patients (5.9 per cent) exposed to thyroid surgery or surgery and x rays and in 1 out of 36 patients (2.8 per cent) tested prior to thyroid injury. These relative frequencies are not significantly different and the frequencies 5.6 or 5.9 per cent are not higher than the incidence of thyroglobulin auto antibodies in ordinary patients without thyroid disease which in Europe varies from 5 per cent (24) to 11 per cent (12). In only 1 out of 23 patients a negative reaction previous to treatment was followed by a positive after treatment. Since this patient (Case 2) had a metastatic infiltration of his thyroid from a tracheal carcinoma the appearance of thyroglobulin auto-antibodies may have been unrelated to surgery or x rays. Consequently the danger of auto antibody formation against thyroglobulin by the described types of surgical and radiologic injury to normal human thyroids seems to be very small.

One of the patients (Case 1) developed hypothyreosis following x ray treatment of a laryngeal carcinoma. At the same time a high

titer of thyroglobulin auto-antibodies (1:25 000) was found. Although patients with post-irradiation myxoedema more frequently contain thyroglobulin auto-antibodies than postirradiation euthyretic patients (2), the cause of this coincidence remains obscure. The explanation may be that such patients prior to treatment already had a latent or unapparent autoimmune reaction towards their thyroid which was enhanced by radiation and provoked myxoedema (18). This hypothesis may also explain why ionizing radiation on thyrotoxic glands (18) in contrast to radiation on normal thyroid glands, as shown by the present study, frequently initiates thyroglobulin auto-antibody production.

Hjort (10) has shown that thyroglobulin gains entrance to the circulation after surgical injuries of the thyroid gland. Robbins (21) and Robbins *et al* (22) could detect circulating thyroglobulin after administration of therapeutic doses of I^{131} . Lindsay *et al* (15) observed pathological changes in thyroids consisting of follicular atrophy and fibrosis in patients treated with x-rays for laryngeal cancers. One of the present patients developed myxoedema subsequent to x-ray treatment of a laryngeal cancer. Consequently, external x-rays employed for treatment of tumors of the laryngeal region do affect the thyroid gland. Furthermore, ionizing radiation as well as surgical damage, apparently injure the thyroid in such a way that thyroglobulin gains entrance to the circulation. But this leakage does not seem to stimulate thyroglobulin auto-antibody synthesis. On this basis the present results represent evidence against the "leakage" theory.

The present results are in agreement with those of Hjort (10), who detected thyroglobulin antibody after exposure to thyroid surgery in only 1 of 29 patients with thyroid disease who prior to surgery had not had thyroglobulin auto-antibody in their serum. Furthermore the "leakage" theory is not supported by experimental investigations. Intravenous injections of thyroglobulin do not elicit antibody formation (25) and a destruction of thyroid structure by I^{131} with an expected leakage of thyroglobulin does not stimulate thyroglobulin auto-antibody synthesis (27).

Consequently, the production of thyroglobulin auto-antibody seems to imply a breakage of tolerance either by an alteration of the antigen itself (hypothesis 2 A) or it may be secondary to infiltration of the thyroid gland by lymphoid cells (hypothesis 2 B). Our Case 2 may support the latter hypothesis. Since x-rays and thyroid surgery do not seem to induce thyroglobulin auto-antibodies, this patient may have started to produce such antibodies as a consequence of the neoplastic invasion of the thyroid. The explanation may be that the lymphoid cells which surrounded the malignant cells in the thyroid have initiated thyroglobulin auto-antibody synthesis after contact with thyroid elements. Since apparently not all kinds of lymphoid cell infiltration of the thyroid do elicit thyroglobulin auto-antibodies (25, 27), some spe-

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A METHOD FOR QUANTITATIVE EVALUATION OF THE NUCLEAR DISTRIBUTION IN THE GLOMERULI OF RENAL BIOPSY SPECIMENS

By

ARNE W S SORENSEN

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In 1936, *Bell* (1) described a method for the quantitative evaluation of a so-called "glomerulitis", by which he understood hypercellularity in a glomerulus. *Bell* drew all the endothelial and epithelial cells in the glomerular tufts in histological sections of the kidney, and found among other things that the ratio of endothelial cells to epithelial cells in the glomerular tufts was less than one in 84.1 per cent, equal to one in 15 per cent and greater than one in 0.9 per cent, in a material of 107 individuals who had died following an accident. The ratio equal to one was described as "glomerulitis" group 0, and ratios greater than one were graduated as "glomerulitis" group 1, 2, 3, etc., dependent on the magnitude of the figure.

In the following, a more simple method is described for evaluation of the total number of cell nuclei in the glomerular tuft (endothelial and epithelial cells), per unit of area in a histological section of a kidney biopsy specimen.

The original reason for the employment of the method was the wish to evaluate the number of cell nuclei in the glomerular tuft in a material of 28 patients with rheumatoid arthritis compared with the number of cell nuclei in a normal control group of 11 patients. This study will therefore be briefly described as an example of the utility of the method (*Brun et al* 1965 (3)).

MATERIAL AND METHODS

The normal control group comprises renal biopsies from 11 patients (7 women and 4 men) the mean age being 46 years (27-60 years). In these patients a peroperative transperitoneal renal biopsy was performed in connection with operation for gallstone. The type of biopsy needle was the same as that used for the percutaneous biopsies (*Liersen & Brun* 1951 (5) and *Brun & Raaschou* 1953 (2)). The patients had normal endogenous creatinine clearance and Addis Shevky's concentration test no proteinuria normal blood pressure and they had not previously had symptoms of diseases of the kidney or urinary tract. The histological sections of the

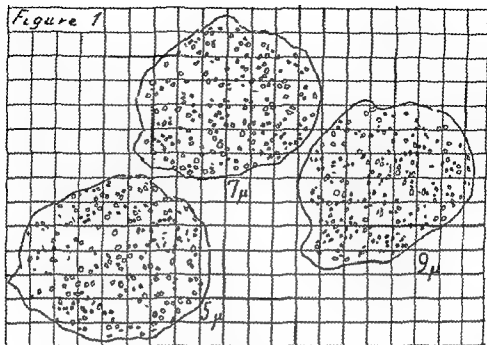


Fig 1

The figure shows 3 neighbouring sections of the same glomerulus in a control biopsy, the individual thickness being 5 7 and 9 μ respectively, drawn by the technique described in the text. The drawing is divided into standard squares of 625 μ^2

biopsy specimens were described as normal on examination by light microscopy.

The kidney tissue obtained was fixed embedded in paraffin and cut in 5 μ sections, subsequently the section thickness was measured optically in the microscope by focussing on lower and upper surfaces (Pearse 1961 (4)). The quantitative evaluation was carried out on sections stained with haematoxylin and eosin.

The cell nuclei in the glomerular tuft were counted by means of a microscope with a drawing device. The glomeruli were projected on to the paper and a line drawn corresponding to the inside of Bowman's capsule.

All cell nuclei in the glomerular tuft were then drawn and counted (Fig 1). Only the well preserved glomeruli were recorded. The area of the single glomerulus was measured by means of a planimeter and the values for each biopsy were summed up.

The significance of variations in thickness of the preparations was examined with a view to the influence on their mutual comparability. If the section thickness is equal to or less than one nuclear diameter more than one whole cell nucleus cannot be present at any one point of the section. Within this limit therefore minor variations in section thickness have no significant influence on the number of nuclei in the section. This is illustrated in the following way. Fig 2 shows one of four drawings consisting of equally large circles of diameter d at the same mutual distance a which on the four drawings is equal in turn to $0.5d$ ($i.e.$ the circles touch each other), $0.5d$, $1.0d$ and $1.5d$ respectively. On each drawing 10 "sections" (parallel lines of constant length) are drawn at random to give a thickness of $0.5d$, $0.7d$, $0.9d$, and $1.1d$. The number of circles intersected by each section is counted and the mean value and variance calculated. Table 1 shows the significance of the difference in number of circles intersected in sections of different thickness in each drawing. Variations in section thickness within the value of the circle diameter are seen to have no or little significance.

In the biopsies the maximum diameter of the nuclei (equatorial sections) were

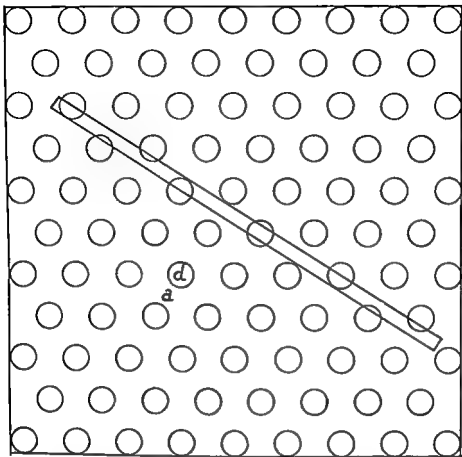


Fig 2

The figure shows uniform circles with a diameter of d . The distance (a) between 2 neighbouring circles $= d$. A section of thickness $= 0.5 d$ lies across the drawing. See text.

found to be 8.10μ and the distance between the nuclei varied from zero up to four or five times the diameter of the nucleus.

In order to test the above argument the cell nuclei were counted in three neighbouring sections of the same three randomly chosen glomeruli in a biopsy from one of the controls; the sections being 5.7 and 9μ thick, respectively. Table II shows the nuclear counts in the three glomeruli in the different section thickness. No significant variation is found in the nuclear counts for the individual glomeruli when the section thickness changes from 5.9μ .

RESULTS

The count method was applied for two purposes, which will be discussed here as a practical application.

1) to determine the mean number of nuclei per unit area of the glomerular tuft ($1000 \mu^2$) which for the eleven patients was 7.8 ± 1.2 .

nuclei (mean value \pm standard deviation) In the group of 28 patients with rheumatoid arthritis, where kidney biopsy was performed percutaneously, the mean value was 70 ± 17 in 15 patients without proteinuria and with normal serum creatinine and 77 ± 15 in 13 patients with elevated serum creatinine and/or proteinuria These values do not differ significantly

TABLE 1

Relationship between Thickness of Section and Number of Circles which are Intersected by the Section on the Drawings

Drawing	Circle diameter	Circle distance (a)	Significance of difference in number of circles intersected in sections of different thickness as compared with a section of thickness 0.5 d		
			0.5/0.7 d significance*	0.5/0.9 d significance*	0.5/1.1 d significance*
1	d	0 d	—	—	+
2	d	0.5 d	—	+	+
3	d	1.0 d	—	—	+
4	d	1.5 d	—	—	+

* — = no significance + = significance ($p < 0.05$) See text

TABLE 2

Number of Cell Nuclei from 3 Randomly Selected Glomeruli in 3 Adjacent Sections of Different Thickness in a Biopsy from the Control Group

Section thickness μ	Glomerulus No 1	Glomerulus No 2	Glomerulus No 3
5	163	212	185
7	153	208	187
9	168	219	197

2) To estimate whether localized accumulations of cell nuclei might be present in the glomeruli in patients with rheumatoid arthritis as it often had been our impression that this might be the case in some glomeruli

To elucidate the problem of a possible localized accumulation of cell nuclei in the glomeruli, the glomerular cell nuclei have been counted in a standard square with a side of 25μ (Fig 1) This area has been selected since the possible histological element appeared to be of this order of magnitude

By examination of the distribution of the nuclear count per unit area ($625 \mu^2$) it is possible to show whether or not the cell nuclei occur

in localized accumulations. If there is a localized accumulation of cell nuclei in the glomeruli, the number of squares containing many nuclei will be increased in comparison with the distribution found in the control group. Likewise, the number of squares with no or few cell nuclei will also be increased, since it was found that the total number of nuclei per unit area remained unchanged from the rheumatoid arthritis group to the control group.

By means of statistical analysis (probit diagrams), the number of cell nuclei per square of $625 \mu^2$ were found to be distributed in a uniform pattern in biopsies from the eleven controls. In the rheumatoid arthritis group the spread of values obtained in the individual biopsies was slightly greater, and besides the variation in the spread between the individual biopsies was slightly greater than that in the biopsies obtained in the control group.

On this basis it was decided to compare the ratio between mean (\bar{x}) and variance (s^2) for each biopsy (s^2/\bar{x}) as the shape of the curve in the probit diagrams recalls the binomial or Poisson distribution. Calculation showed that this ratio on an average was about 0.75 for the control biopsies. For the biopsies in the rheumatoid arthritis group it was significantly higher (Wilcoxon's rank test). For further references see Brun *et al* (1965 (3)).

A localized accumulation of cell nuclei in the glomeruli is seen in patients with rheumatoid arthritis but not in the controls. But the phenomenon was observed only in connection with other histological lesions in the kidneys (amyloidosis, chronic interstitial nephritis) and was not observed in otherwise normal kidney biopsies from these patients.

DISCUSSION AND CONCLUSION

In a given specimen of renal tissue (kidney biopsy or autopsy specimen) it may be very difficult to determine whether it is a matter of diffuse glomerular hypercellularity (diffuse glomerulitis) or of a localized accumulation of cells (localized glomerulitis).

The above mentioned method has proved to be applicable for the solution of a concrete problem: Have patients with rheumatoid arthritis diffuse and/or localized hypercellularity in the glomerular tufts in the kidneys, in contrast to the controls?

The reason why the method has been described as a simple method is that it is easy to carry out, unfortunately, however, it is troublesome and very time consuming. On the other hand, the method makes it possible to photograph suitable glomeruli in a given specimen instead of drawing them to count the cell nuclei, measure the area of the glomerulus on the photo under standard conditions, and compare the result with the average number of cell nuclei per unit area (e.g. $1000 \mu^2$) in a normal control preparation of reasonable magnitude.

nuclei (mean value \pm standard deviation) In the group of 28 patients with rheumatoid arthritis where kidney biopsy was performed percutaneously, the mean value was 70 ± 17 in 15 patients without proteinuria and with normal serum creatinine and 77 ± 15 in 13 patients with elevated serum creatinine and/or proteinuria These values do not differ significantly

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1	d	0 d	—	—	+
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The reason why the method has been described as a simple method is that it is easy to carry out, unfortunately however it is troublesome and very time consuming. On the other hand the method makes it possible to photograph suitable glomeruli in a given specimen instead of drawing them. To count the cell nuclei measure the area of the glomerulus on the photo under standard conditions and compare the result with the average number of cell nuclei per unit area (e.g. $1000/\mu^2$) in a normal control group of the same magnitude.

SUMMARY

This paper provides a description of a method for quantitative evaluation of the number of cell nuclei in the glomerular tufts in kidney biopsy specimens

The application of the method is illustrated by an example. An evaluation is made to see whether patients with rheumatoid arthritis have a diffuse and/or localized hypercellularity in the glomerular tufts of the kidneys. The method has proved to be applicable to this concrete problem, but in a modified form it may be possible to apply it generally to selected problems. On the other hand, the method cannot be recommended as a routine, as it is time consuming and troublesome.

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Methods a and b seemed to give comparable coating of the rods, whereas method c may be less reliable because droplets of vaseline set on the "edges". Method a or b was used in the majority of experiments carried out.

In our experiments, the material sampled from air was transferred to culture medium by pulling the rod over a plate, simultaneously performing zig zag movements. If a heavy harvest of microbes is to be expected, it is necessary to pull the rod over 2-3, or even more, consecutive plates in order to obtain a quantitative measure of the air contamination. The choice of medium is dependent upon the species to be isolated. In our experiments we used t-3ptose agar (Difco) with 10 per cent blood.

TABLE 1

Comparative Sampling with Roto Rod Sampler and Exposed Open Blood Plates

Dates of sampling 1965	Roto-Rod Sampler					Exposed open blood plates				
	Colonies sampled per minute, equal 40 litres of air					Mean numbers of colonies per plate per minute exposed				
	Hours					Hours				
	07 30	10 00	12 00	15 00	17 00	07 30	10 00	12 00	15 00	17 00
20 Sept	65.2	73.2	45.2	134.0	31.3	6.67	2.00	1.00	6.00	1.33
21 "	216.0	69.6	144.7	61.3	31.6	25.00	1.80	3.00	3.03	0.50
22 "	106.4	29.0	60.5	260.0	34.2	1.48	0.45	1.68	16.41	0.91
27 "	106.4	74.0	164.8	182.2	27.0	6.23	1.88	1.28	2.09	0.77
28 "	334.6	53.4	67.2	102.2	22.6	9.01	1.79	0.75	2.94	0.29
29 "	90.8	112.8	96.6	271.0	78.0	6.63	2.73	1.40	14.93	1.11
4 Oct	73.6	65.6	67.0	500.2	46.8	1.13	3.11	1.05	8.18	0.58
5 "	222.2	110.4	156.0	689.2	100.0	2.30	1.03	1.20	8.00	0.93
6 "	95.0	360.2	151.4	569.2	88.6	1.59	3.63	1.20	10.17	1.17
11 "	399.4	112.4	12.4	132.8	25.2	5.99	0.53	0.23	1.07	0.31
12 "	593.6	68.6	68.8	195.0	33.0	8.67	0.66	0.61	4.57	0.36
13 "	216.0	44.2	13.2	51.8	52.2	2.21	2.83	0.55	0.91	0.77
Sum	2515.7	1173.4	1027.8	3348.9	570.5	77.01	22.44	12.27	78.29	9.03
Mean	209.8	97.8	85.6	279.1	47.5	6.40	1.90	1.00	6.50	0.75
Mean per litre	5.25	2.45	2.14	6.98	1.19					

A pilot series of experiments were carried out in a comparatively large laboratory in the department of bacteriology, with the Roto-Rod Sampler placed on a shelf in the middle of the room, 1.60 m above floor level. All four "edges" of the rod were coated with vaseline, and the apparatus was run 10 minutes clock wise, and immediately switched over and run 10 minutes counter clock-wise. The harvest was very poor, only a few colonies of micrococci and *B. subtilis* were found, a result which have been rather confusing if we had not as a control measure exposed plates of medium at the same time and the same place. Having completed the pilot study in the laboratory, the Roto Rod Sampler was tested in various departments of the hospital. Particular attention was paid to the handling of used bed linen, which was checked step by step from bed changing until it was placed in the washing machine. Variable amounts of microbes were sampled by the rod, and pathogenic

at 15⁰⁰ just after the beds were smothered out after visiting time and the final fifth sampling was timed at 17⁰⁰ when little or nothing was going on in the room. The sampling was carried out over periods of three consecutive days in all 4 periods.



Fig 1

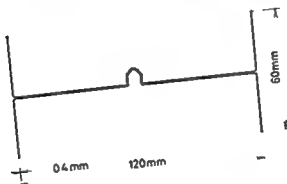


Fig 2

Form and dimensions of the Rod

- 2 To perform comparative sampling with the Roto Rod Sampler and exposed open plates of medium

The "edges" of the rod must be coated with a thin layer of some sticky medium. For their particular purpose the expert of the mentioned research establishment used silicon grease. Being ignorant of the tolerance of microbes to silicon grease we selected sterilized vaseline for the coating of the "edges" of the rods.

- The rods were coated with vaseline in one or another of the following ways
- a Sterile melted vaseline was spread over a flamed knifeblade and the "edges" of the rod were rubbed over the blade
 - b Instead of a knife blade the vaseline was spread over a sterile glass plate and the rods were processed as mentioned under a
 - c The edges of the rods were dipped in melted vaseline

Methods a and b seemed to give comparable coating of the rods whereas method c may be less reliable because droplets of vaseline set on the 'edges'. Method a or b was used in the majority of experiments carried out.

In our experiments the material sampled from air was transferred to culture medium by pulling the rod over a plate simultaneously performing zig zag movements. If a heavy harvest of microbes is to be expected, it is necessary to pull the rod over 2-3 or even more, consecutive plates in order to obtain a quantitative measure of the air contamination. The choice of medium is dependent upon the species to be isolated. In our experiments we used tryptose agar (Difco) with 10 per cent blood.

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Sum	2515.7	1173.4	1027.8	3348.9	570.5	77.01	22.44	12.27	78.21	9.61
Mean	209.8	97.8	85.6	279.1	47.5	6.40	1.90	1.00	6.50	1.15
Mean per litre	5.25	2.45	2.14	6.98	1.19					

A pilot series of experiments were carried out in a comparatively large building in the department of bacteriology with the Roto-Rod Sampler placed on a table in the middle of the room, 1.60 m above floor level. All four 'edges' of the plate were coated with vaseline, and the apparatus was run 10 minutes clock wise and then directly switched over and run 10 minutes counter clock wise. The harvest was poor, only a few colonies of micrococci and *B. subtilis* were found, a result which may have been rather confusing if we had not as a control measure exposed a blood plate in the laboratory at the same time and the same place. Having completed this experiment in the laboratory the Roto Rod Sampler was tested in various departments of the hospital. Particular attention was paid to the handling of used bed linen. It was checked step by step from bed changing until it was placed in the disposal machine. Variable amounts of microbes were sampled by the rod. *Staphylococci* were demonstrated all along the route.

In a four bed room in the surgical department sampling was carried out five times daily. The first sampling took place at 7.30 just after the morning ward round. The second and third samplings were performed at 10.00 and 12.00, respectively, which represents a comparatively quiet time in the ward. The fourth sampling was at 15.00 just after the beds were smoothed out after visiting time. The fifth sampling was timed at 17.00 when little or nothing was going on. The sampling was carried out over periods of three consecutive days.

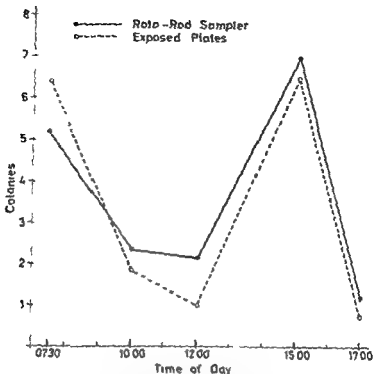


Fig 3

Number of colonies (microbes) sampled with the Roto Rod Sampler from per liter air examined compared with the mean number of colonies (microbes) per minute per exposed open plate.

In all experiments the Roto Rod Sampler was compared with 10 exposed open blood plates. The 3 first days the Roto Rod Sampler was run for 10 minutes and the following days for five minutes. The material collected on the "edges" of the rods were transferred to plates as described above. The first five sampling days the plates were exposed for 1/2 hour the following sampling days for 15 minutes.

RESULTS

The results of these experiments are shown in Table 1. They confirm what was already known, that the contamination of the air is a function of activity in the room. Regarding the comparative sampling, there are singular days and hours when results obtained with the Roto-Rod Sampler and the exposed plates seem to disagree. However, on the whole, the average of the results obtained with the Roto-Rod Sampler and the exposed plates compare very well. This is nicely demonstrated in Fig 3 where the number of colonies per liter air sampled with the Roto-Rod Sampler are compared with the mean number of colonies per plate per minute exposed.

When testing the Roto-Rod Sampler we were faced also with the question how to determine the optimal length of time to run the rod

TABLE 2

Results of one, two and five Minutes Consecutive Sampling with Roto Rod Sampler

Sampling time No of tests	1 minute Side		2 minutes Side		5 minutes Side	
	a	b	a	b	a	b
1	17	18	25	12	28	28
2	18	15	16	29	40	23
3	20	18	53	21	40	44
4	27	47	63	24	46	50
5	95	20	39	74	27	48
6	171	51	10	109	26	25
7	13	12	51	49	155	135
8	3	11	23	17	34	59
9	7	1	7	19	37	29
10	27	9	24	25	47	41
	403	202	311	319	480	482
Sum a + b	605		630		962	

TABLE 3

Phage Types of 64 Strains of Staphylococcus aureus

Number	Groups	Types
11	III	83 A
11		77 Ad B5 D
10	VI	82 47 +
2	II	3 A/3B
11	MI	80 kS6 82 6,7 47 53 34 70 73 77 +
1	MI	82 42E, 47 34 70 73 77
1	MI	82 53 +
1	MI	82 83 A
1	I	29 32 79 80 kS6
1	I	52
1	IV	42D
22		N T

It was not unlikely that the high speed of the rod might interfere with the vaseline coating on the "edges" in such a way that the ability to take in microbes might deteriorate.

A preliminary study of this problem is presented in Table 2.

Three rods were run clock wise in consecutive order, the first for one minute, the second for two minutes, and the third for five minutes. The two "edges" coated with vaseline were marked a and b, and the material was transferred to blood plates as described above.

The results of 10 experiments show considerable variation in the number of microbes sampled with "edges" a and b in the individual experiments. Further, the results seem to indicate that the number of

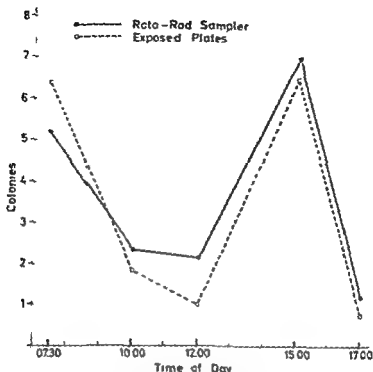


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The results of these experiments are shown in Table 1. They confirm what was already known, that the contamination of the air is a function of activity in the room. Regarding the comparative sampling, there are singular days and hours when results obtained with the Roto-Rod Sampler and the exposed plates seem to disagree. However, on the whole, the average of the results obtained with the Roto-Rod Sampler and the exposed plates compare very well. This is nicely demonstrated in Fig 3 where the number of colonies per liter air sampled with the Roto-Rod Sampler are compared with the mean number of colonies per plate per minute exposed.

When testing the Roto-Rod Sampler we were faced also with the question how to determine the optimal length of time to run the rod

The Institute of Clinical Bacteriology (Head Prof Sten Winblad M.D.)
University of Lund, General Hospital, Malmö, Sweden

STUDIES ON *YERSINIA ENTEROCOLITICA*

Characterization of 28 Strains from Human and Animal Sources

By

BIRGITTA NILEHN

Received 31.1.66

Investigations of biochemical, physiological, and serological properties of the species *Yersinia enterocolitica* (Y₄), have been published by several authors (Hässig *et al* 1949, Dickinson & Mocquot 1961, Becht 1962, Daniels & Goudzwaard 1963, Daniels 1963, Akkermans & Terpstra 1963, Knapp & Thal 1963, Siegmänn 1963, Mollaret & Chevalier 1964, Mollaret & Lucas 1965, Frederiksen 1964, Carlsson *et al* 1964, Smith & Thal 1965, Wauters & Mollaret 1965, Winblad *et al* 1966). Five related bacterial strains were published under the name of *Bacterium enterocoliticum* by Schleifstein & Coleman 1939.

However, most of the strains hitherto described have apparently been isolated from animal sources.

Isolation of such strains from man has only occasionally been reported (Schleifstein & Coleman 1939, Hässig *et al* 1949, Carlsson *et al* 1964, Mollaret & Chevalier 1964, Wauters & Mollaret 1965, Winblad *et al* 1966). Judging from preliminary investigations, Y₄ is a fairly common human pathogen (Winblad, Nilehn & Sternby).

The purpose of this paper is to present some characteristics of strains recently isolated from human sources. Seventeen of the strains in this study have been isolated from patients, all of whom except one, had had acute abdominal symptoms, occasionally also diarrhoea. Two of the cases have been reported by Winblad *et al* (1966). Some older strains from various sources were also included in the tests. Most of the tests were carried out at 37° C as well as at 22° C.

MATERIAL AND METHODS

Bacterial strains. Eleven Y₄ strains from different sources and previously described by Hässig *et al* (1949), Becht (1962), Daniels & Goudzwaard (1963), Daniels (1963), Knapp & Thal (1963), Siegmänn (1963), Mollaret & Chevalier (1964), Frederiksen (1964), Carlsson *et al* (1964), Smith & Thal (1965).

microbes sampled do not increase proportionally with prolonged running time

By the studies reported no attempt was made to identify every species taken in by the Roto-Rod Sampler. However, in all series of experiments, the cultures were searched for pathogenic staphylococci, and the morphology of other microbes were determined. The main lot of microbes was cocci—less frequently rods (*B. subtilis*, *E. coli*, *Achromiobacter*).

Strains of *Stph aureus*, coagulase positive, were isolated in all experiments carried out in the wards. Of all strains of staphylococci sampled in the four-bed-room, (the material reported in Table 1) 64 were typed by phages. The groups and types presented in Table 3, cover a wide spectrum which may indicate inflow from varied sources. The one strain of group IV, 42 D, was the only one isolated from material sampled from the hospital during 1965.

CONCLUSION

Our conclusion, based on the preliminary experiments carried out with the Roto-Rod Sampler, indicates that this handy apparatus might be of great value in all efforts to investigate and control microbial air contamination.

Further detailed studies of particular problems connected with the use of the Roto-Rod Sampler—such as coating of the "edges", optimal running time, and transfer of the material to suitable medium are needed.

For testing of sensitivity to antibiotics human blood agar without peptone added
LSU agar (Juhlin & Ericson 1961)

Tests for identification

Gram staining Smears were made from 24 and 48 hour cultures on human blood agar

Oxidation/fermentation test Unless otherwise stated 0.5 per cent concentrations of respective test substances in liquid base medium were used in the tests

Base medium Peptone 1 per cent Meat extract 0.5 per cent NaCl 0.5 per cent Bromthymol blue in absolute alcohol 0.0024 per cent Final pH 7.5-7.6 Medium without test substance was used as control

The tubes were inoculated from human blood agar cultures (24 hours 37°C) with a platinum loopful of the culture and incubated at 22°C and 37°C respectively

Quantitative determination of the inoculum was not performed repeated test series however always gave identical results The tubes were inspected after 1 2 3 5 10 20 and 30 days' incubation

Test substances L-arabinose (Brit Drug Houses Co Ltd Lond 2390340) L-rhamnose (Hecho AB Stockholm Sweden 55100) D-xylose (F. Merck AG Darmstadt Germany 8092) D-glucose (Baker Chem Co Phillipsburg N.J. 1916) D-fructose (Hecho AB S 5043) D-galactose (Merck 4061) D-mannose (1 per cent) (Pfannstiehl Lab INC 1219 Glen Rock Ave Waukegan Cleveland Ohio) lactose (Baker Chem Co 2248) sucrose (Baker Chem Co 4072) maltose (Merck 5910) raffinose (Nutritional Biochemicals Corporation Cleveland Ohio 1-1109), D-trehalose (hydrate)

Grave
per cent
redistilled

Corporation 12341) salicin (Nutritional Biochem Corporation S 3771)

Aesculin splitting was tested according to Cowan & Steel 1965 (Aesculin Merck 5788 5325)

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L-proteose peptone No. 3 0065 01 (Difco Lab Detroit Michigan) 2 per cent dipotassium phosphate 0.1 per cent sodium thiosulphate 0.00003 per cent agar 1.5 per cent distilled water, pH 6.7 The medium was tiled and stab inoculated Readings after 1 2 3 5 10 20 and 30 days

Decarboxylase reactions according to Møller (1955) L-ornithine monohydrochloride 12.750 and L-arginine monohydrochloride (Nutritional Biochemicals Corporation) L-lysine monohydrochloride CHR (Fluka AG Chemische Fabrik Buchs SG Switzerland) were used as test substances Medium without test substance was included in the tests

Phenylalanine deamination according to Shaw & Clarke (1955) (DL-phenylalanine CHR 46629 Fluka AG Buchs SG Switzerland) Incubation time 2 days

Citrate utilization according to Cowan & Steel (1965) medium modified from Auster (1923)

Voges-Proskauer (V-P) reaction according to Barritt (1936) slightly modified The method will be described in detail in a future paper

Media were made from the test tubes
ants were investigated in a shortened
Gram stained smears and registration
slide agglutination and tests for acid

formation from lactose D-mannitol and sucrose

Motility studied in 0.2 per cent agar in base medium in 1 tubes

Incubation was done in incubators at 22°C ± 1°C and 37°C ± 0.5°C The temperature was continuously registered with a thermograph

Slide agglutination was done with rabbit antisera prepared against O antigen and

Strains ¹	Original no	Source
334	Siegmann 268	Chinchilla
335, 337, 338 339	Daniels 1975 1905 924 931	Chinchilla
336	Hassig	Homo
347, 348	Becht 18 51	Chinchilla
356	Becht 200	Dog
357	Frederiksen P71	Chinchilla
PA	Winblad	Homo

Strains recently isolated from human sources

6498	Isolated from appendix Patient female, 24 years	Diagnosis acute terminal
	<i>Heilis Mesenteric lymphadenitis</i>	
4528	Isolated from appendix Patient female 8 years	Diagnosis acute terminal
	<i>Heilis Mesenteric lymphadenitis</i>	
5525	(5787, 5867) Isolated from appendix respectively faeces Patient female 29 years, with symptoms suggesting acute appendicitis	
5674	(5722) Isolated from faeces Patient female, 63 years with diarrhoea abdominal pain fever and acute arthritis	
5653	Isolated from appendix Patient male, 24 years	Diagnosis acute terminal
	<i>Heilis Mesenteric lymphadenitis</i>	
5788	(5845, 5846). Isolated from faeces Patient male 51 years with symptoms suggesting acute appendicitis	
5944	(0024) Isolated from appendix respectively faeces Patient female 8 years	Diagnosis acute terminal
	<i>Heilis Mesenteric lymphadenitis</i>	
6064	(278, 430 503 489) Isolated from appendix respectively faeces Patient female 21 years	Diagnosis mesenteric lymphadenitis
144	(146 101) Isolated from faeces Patient female 20 years with acute abdominal pain	
504	(754 784) Isolated from appendix respectively faeces Patient male 14 years	Diagnosis mesenteric lymphadenitis
582	Isolated from faeces Patient female 36 years hospitalized because of high l S R	
953	(1112) Isolated from faeces Patient female 6 years acute abdominal pain	
1029	Isolated from appendix Patient male 19 years	Acute appendicitis
1077	Isolated from faeces Patient male 11 years	Acute abdominal pain
1168	Isolated from faeces Patient female 22 years hospitalized because of diarrhoea arthralgia and erythema nodosum	
1691	Isolated from appendix Patient female 46 years Operated upon because of left sided ovarian cyst acute abdominal symptoms	
1769	Isolated from appendix Patient male 24 years Symptoms suggesting acute appendicitis	

Figures in brackets denote code numbers of repeated isolates from the same patient

Primary isolation was done at 22° C. and in some cases also at 37° C. on LSL agar (Juhlin & Ericson, 1961)

Pure cultures of both primarily isolated strains and of older strains were obtained by repeated subculture of apparently pure colonies at 37° C. on LSL agar and human blood agar. The strains were then primarily tested regarding their biochemical physiological and serological properties and for their sensitivity to antibiotics.

The strains were stored on human blood agar at 22° C. for longer intervals on human blood agar at +5° C. with subculture every 3rd to 4th week.

As soon as pure cultures had been obtained after primary isolations the strains were also freeze dried to ensure material in case of contamination or for supplementary examinations.

Solid Media Used

Human blood agar (with 1 per cent Proteose peptone no 3 Difco Lab Detroit Michigan)

¹ Strains 334-357 were kindly placed at our disposal by Prof. E. Thal, The State Veterinary Medical Institute, Stockholm.

For testing of sensitivity to antibiotics: human blood agar without peptone added
LSII agar (Juhlin & Ericson 1961)

Tests for identification

Gram staining. Smears were made from 24 and 48 hour cultures on human blood agar

Oxidation/fermentation test Unless otherwise stated, 0.5 per cent concentration of respective test substances in liquid base medium were used in the tests

Base medium: Peptone 1 per cent, Yeal extract 0.5 per cent, NaCl 0.5 per cent Bromthymol blue in absolute alcohol 0.0024 per cent Final pH 7.5-7.6 Medium without test substance was used as control

The tubes were inoculated from human blood agar cultures (24 hours, 37° C) with a platinum loopful of the culture and incubated at 22° C and 37° C respectively.

Quantitative determination of the inoculum was not performed, repeated test series however, always gave identical results. The tubes were inspected after 1, 2, 3, 5, 10, 20, and 30 days' incubation.

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Corporation 12341), salizin (Nutritional Biochem Corporation S 3771)

Aesculin splitting was tested according to Cowan & Steel 1965 (Aesculin Mersch

according to *Bailou*
* 28, 5653, 5788, 5525
Sigma Chemical Com

Catalase reaction (slide method) H₂O₂ 3 per cent in distilled water

1 2 3, 6 10 20 and 30 days

in the tests

Phenylalanine decarboxylation according to Shaw & Clarke (1955) (DL-phenylalanine CIBR A6628 Fluka AG Buchs SG Switzerland) Incubation time 2 days.

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The method will be described in detail in a future paper.

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formation from lactose and mannitol and sucrose

Mortality studied in 02

Incubation was done in

Side agglutination was

OH antigen preparations as well as against suspensions of living bacteria of a known Y strain (P\N) according to Winblad *et al* (1966)

Tube agglutination was done with the use of rabbit O antisera against strain P\N and O antigen preparations of the strains in question according to Winblad *et al* (1966) As O antigen suspensions containing about 10⁸ bacteria/ml were used in the tests

Tests for sensitivity to antibiotics were performed according to Friesson (1960, 1966)

Filter paper discs with antibiotics and data regarding the regression lines were obtained from Bacteriological Department, Karolinska sjukhuset, Stockholm Sweden

Discs containing the following substances were used Benzyl Penicillin (20 IU) Methicillin (30 mcg), Ampicillin (20 IU), Streptomycin (50 mcg), Tetracycline (50 mcg), Oxytetracycline (50 mcg) Chloramphenicol (30 mcg), Kanamycin (5 mcg), Erythromycin (50 mcg), Nitrofurantoin (30 mcg) Neomycin (50 mcg), Sulphonamide (Ithosin) (2.4 mcg), Colistin (900 IU) Salicylic acid (30 mcg), Fucidin (50 mcg) Novobiocin (50 mcg), Oleandomycin (50 mcg)

The bacteria were suspended in saline and seeded on blood agar plates so as to give dense but not confluent growth on the plates Incubation at 37° C over night

The inhibition zones were measured in mm and the sensitivity was calculated according to regression lines for each antibiotic as determined by Friesson (1960, 1966) The sensitivity was graded as follows I = "Sensitive", II = "Fairly sensitive", III = "Slightly sensitive", and IV = "Resistant"

RESULTS

Morphology

Gram-stained smears from recently isolated Yc cultures plated on human blood agar showed Gram negative rods with small coccoid forms dominating

Diagnostic Tests

Results of oxidation/fermentation tests at 22° C and 37° C Recently isolated human strains attacked the carbohydrates tested in a very uniform way Thus all formed acid rapidly (1-2 days) from d glucose, d-fructose, d mannose, sucrose, d trehalose, sorbitol d-mannitol at both temperatures No acid was formed from l-rhamnose, lactose, d-raffinose, inulin, dulcitol, adonitol, and salizin The results obtained from l-arabinose, d-xylose d galactose maltose and d glycerol are presented in Table 1

Acid formation was seen somewhat slower (2-5 days) from d-galactose, and from l-arabinose The results obtained with maltose differed with the incubation temperature, acid being formed rapidly at 22° C but late (10-30 days) or not at all at 37° C Acid was also formed rapidly from glycerol at 22° C but somewhat later (2-5 days) at 37° C

The old strains of animal origin behaved in the same way as the human strains except concerning d-xylose At 22° C all chinchilla strains formed acid rapidly from this sugar but rather late at 37° C Both recently isolated and old human strains as well as the dog strain showed negative or late and irregular acid production

Slight gas production at 22° C was noted most distinctly from maltose, d mannitol, d glucose, d-trehalose, glycerol, sorbitol and in some cases also from d-xylose Slight gas formation was also noted (though irregularly) from d-glucose at 37° C

TABLE 1

Acid Formation from l Arabinose d xylose d Galactose Maltose and Glycerol at 22° C and 37° C by 28 strains of V enterocolitica from Human and Animal Sources

Strain.	Origin	l arabinose		d xylose		d galactose		maltose		glycerol	
		22°	37°	22°	37°	22°	37°	22°	37°	22°	37°
6498	man	2	2	—	—	3	3	1	10	1	11
4528		2	2	—	20	3	3	1	20	1	3
5525		2	2	—	—	2	11	2	—	1	5
5674		2	2	—	—	3	11	1	—	1	5
5251		3	3	—	—	1	1	2	—	1	3
5788	"	2	2	—	—	11	1	1	—	1	2
5944		3	1	—	—	3	3	1	—	1	1
6064		11	11	—	—	5	5	2	—	1	3
101		5	5	—	—	3	3	1	—	1	3
564		2	2	—	—	3	3	1	—	1	3
582	"	2	2	—	—	3	3	1	—	1	3
953		3	3	—	—	3	3	1	10	1	11
1029		3	3	—	—	5	11	1	—	1	3
1077		3	3	—	—	5	5	1	—	1	2
1168		3	3	—	—	5	3	2	—	1	3
1891	"	3	3	—	—	3	3	1	—	2	3
1759		2	5	—	—	5	3	1	10	2	3
PV*		2	2	—	20	3	3	1	10	1	5
336*		3	3	—	20	5	11	1	20	1	5
356*		2	2	20	20	3	3	1	10	1	2
334*	dog chinchilla	1	1	2	20	3	3	1	20	1	5
335*		1	1	1	10	3	3	1	—	1	5
337*		1	1	1	20	3	3	1	—	1	5
338*		1	1	2	5	3	1	1	10	1	3
339*		3	3	1	20	3	3	1	—	1	5
347*	"	1	1	2	20	3	3	1	10	1	5
348*		1	1	1	10	3	3	1	20	1	5
357*		1	1	1	10	3	3	1	10	1	1

The figures indicate the day on which acid formation was registered. The tubes were inspected after 1 2 3 5 10 20 and 30 days incubation.

— negative reaction after 30 days * old "laboratory" strains

The V P reaction was positive at 22° C but negative at 37° C for all newly isolated human strains.

Aesculin splitting was negative in all cases except 2 (strains 357 and 335) where a positive reaction was seen at 37° C after 5 and 7 days respectively.

All strains give a positive urease test most rapidly at 37° C.

The OMPG reaction was positive for all strains tested except one (strain 356). Positive reactions were seen earlier at 22° C (after about 3 hours) while at 37° C several strains did not give distinctly positive results before 24 hours in 2 cases (338 5788) not before 48 hours.

The catalase reaction was positive in all cases. The oxidase reaction was negative. All strains reduced nitrate at 22° C and at 37° C. Indole and H₂S were not produced.

Ornithine decarboxylase was invariably positive strongest at 22° C. Arginine and lysine were not decarboxylated.

Phenylalanine was not deaminated, and citrate was not utilized at either temperature.

All strains were motile in semisolid agar at 22° C, but not at 37° C.

In tube test O-antigen preparations of all human strains were agglutinated by rabbit immune serum against strain PX at a titre of 1:640-1:1280. O-antigen preparations of the animal strains were agglutinated at approximately the same titres. Only for one strain (357) the O-antigen preparation was agglutinated at a lower titre (1:80-1:160). All strains were distinctly agglutinated on slide agglutination with rabbit antisera, prepared against O-, OH-, and living antigen preparations of strain PX.

TABLE 2

Sensitivity to Antibiotics of 28 Yersinia enterocolitica Strains from Human and Animal Sources Determined by the Paper Disc Method according to Friesson 1960, 1966

	I	II	III	IV
Streptomycin	29		-	
Tetracycline	28			
Ox-tetracycline	28			
Chloramphenicol	28			
Nitrofurantoin	29			
Colistin	28	-		
Nalidixic acid	29			
Neomycin	25	1		
Sulphonamide	29	-		
Kanamycin	-	28		
Erythromycin	-	-	15	13
Ampicillin	2	1	24	1
Benzylpenicillin	-	-	14	14
Methicillin	-	-	-	28
Oleandomycin	-	-	-	28
Novobiocin	-	-	-	28
Fucidin	-	-	-	28

The figures indicate number of strains within each sensitivity group. I = "sensitive", II = "fairly sensitive", III = "slightly sensitive", IV = "resistant".

Test for Sensitivity to Antibiotics

As shown in Table 2 all strains including the older ones of animal origin showed largely the same pattern of susceptibility to various antibiotics. Thus all strains were sensitive to streptomycin, tetracycline, oxitetracycline, chloramphenicol, nitrofurantoin, colistin, sulphonamide and nalidixic acid. Neomycin gave inhibition zones just below the limit for sensitivity grade I for 3 strains, while the other strains were fully sensitive.

Kanamycin regularly gave inhibition zones corresponding to sensitivity grade II. Sensitivity grade III to erythromycin was seen in 15 cases, 8 of the 13 strains in grade IV showed very small inhibition zones.

All animal strains and 2 recently isolated human strains did not show

any zones for benzyl penicillin while the rest showed small zones corresponding to approximately sensitivity grade III. One strain was resistant to ampicillin the values for the others varied from I-III. All strains were resistant to methicillin, fusidic acid, oleandomycin and novobiocin.

DISCUSSION

In previous reports the small number of *Y. e.* strains of human origin hitherto isolated have not been found to differ substantially from those isolated from various animals (Hassig *et al.* 1949; Carlsson *et al.* 1964; Mollaret & Chevalier 1964). For one strain described by Wauters & Mollaret (1965) however, aesculin splitting was positive. The *B. enterocoliticum* strains reported by Schleifstein & Coleman (1939) were atypical in producing indole. Two old strains of human origin (33f Hassig, P. A. Winblad) were included in the present study.

Differences between strains from different animal species have been reported by Mollaret & Lucas (1965). They found strains from hares to differ in certain biochemical properties from strains of other origins.

In the present study the only noticeably difference in biochemical behaviour between human strains and old chinchilla strains included in the tests was found in d-xylene fermentation. Most of the animal strains formed acid rapidly at 22°C while the recently isolated human strains did not (in a few cases very late at 37°C). It must however be borne in mind that the animal strains used in the tests all were laboratory strains in which behavioural changes might have occurred. Furthermore other investigators have found strains from various animal sources to differ regarding xylene fermentation (Becht 1962; Mollaret & Chevalier 1964; Frederiksen 1964).

An interesting finding was the variation of biochemical activity of *Y. e.* with the incubation temperature. Thus diversity in acid formation with greater activity at 22°C than at 37°C was noted from d-xylene, maltose and glycerol. As a rule, production of gas from several carbohydrates was noted only at 22°C. L-ornithine decarboxylation and β -galactosidase activity likewise seemed to be more pronounced at the lower incubation temperature. Negative V-P reactions at 37°C and positive reactions at 22°C were seen in all recently isolated human strains. A more detailed study of the V-P reaction of *Y. e.* will be the subject of a future paper (Videhn to be published). Variation of the activity of *Y. e.* with the incubation temperature was also observed in the V-P reaction by Dickinson & Mocquot (1961), Frederiksen (1964), Mollaret & Chevalier (1964) and Wauters & Mollaret (1965).

As far as is known the influence of temperature on growth and various metabolic processes of *Y. e.* has not been properly investigated. According to Knapp & Thal (1963) the optimum temperature for growth is 37°C but they gave no data on the generation time or growth curves under different conditions. It is however well known that se-

veral biochemical reactions of bacteria are more intense at temperatures below that for optimal growth.

The urease activity was found to be more pronounced at 37° C. Preliminary investigations indicate shorter survival times of the bacteria at this temperature. This might speak in favour for the existence of a mainly intracellular urease enzyme which is liberated in greater amounts in connection with the disintegration of the cells. However, the optimum temperature for the activity of the urease enzyme of this species is not known.

Aesculin splitting was seen rather late at 37° C but not at 22° C for 2 animal strains while all human strains were negative at both temperatures.

The above differences found at different incubation temperatures may lead to confusing results in investigations of the biochemical properties of *Y. e.* unless the incubation temperature is strictly controlled. On the other hand the results of tests carried out at different well controlled temperatures might be valuable in the diagnose of the species.

As to the serological testing of the strains by agglutination, the purpose of the present study was not a detailed determination of any of the various antigen components. However, at least one O-antigen component was found to be common to all the strains included in the study.

The susceptibility pattern of the strains to antibiotics was largely the same as that reported by other investigators (Frederiksen 1961; Smith & Thal 1965).

SUMMARY

Twenty-eight strains of *Yersinia enterocolitica* 17 recently isolated from human sources and 11 old strains of animal and human origin were tested biochemically, physiologically and serologically as well as regarding their susceptibility to antibiotics.

Differences in the biochemical activity at 37° C and at 22° C were noted in a number of oxidation/fermentation reactions, aesculin hydrolysis, decarboxylase activity, β -galactosidase activity, urease activity and in the V-P-reaction. Recently isolated human strains as well as old human strains differed from old chinchilla strains in respect of d-xylene fermentation.

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STUDIES ON TRANSFORMATION IN *MORAXELLA* AND ORGANISMS ASSUMED TO BE RELATED TO *MORAXELLA*

7 Affinities between Oxidase Positive Rods and Neisseriae as Compared with Group Interactions on Both Sides

By

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Relatively efficient genetic transfer has previously been shown to occur between *Moraxella nonliquefaciens*, *Moraxella bovis* and the serum liquefying, nonhaemolytic moraxellae (Bovre 1965 a, c). A 19116.51 group of *Moraxella nonliquefaciens* like organisms has been described (Bovre 1965 d). In quantitative tests the latter group showed no compatibility with the above mentioned moraxellae. However sensitive tests with continuous DNA exposure revealed low frequent reactions between them.

Low frequent transformation of *Neisseria catarrhalis* with DNAs from *Moraxella nonliquefaciens*, *Moraxella bovis* and *Moraxella liquefaciens* was first reported by Bovre (1963). Catlin (1964) has described transformation reactions of similar order between *Neisseria catarrhalis* and some oxidase positive rods probably belonging to the 19116.51 group (Bovre 1965 d).

Observed transformation between *Neisseria catarrhalis*, *Neisseria caviae* and *Neisseria ovis* (Bovre 1965 b) has made also the latter two species natural objects for studies on transformation between oxidase positive rods and neisseriae.

The present report aims at a comparative synthesis of transformation interactions which all turned out as less pronounced than the ones found between *Moraxella nonliquefaciens*, *Moraxella bovis* and the serum liquefying, nonhaemolytic moraxellae. Quantitative streptomycin resistance transformation will be supplemented with semi quantitative estimates with the same marker and a system of transformation with haemolysis as the genetic marker will be introduced.

MATERIAL AND METHODS

All bacterial strains used have been described in previous reports (Bovre 1964 b, 1965 a, b, c). Individual strains were chosen in accordance with results of trans-

formation experiments (*loc cit*) to secure that they were representative of the various groups in question

The methodology of quantitative streptomycin resistance transformation was that initially described (*Boore 1964a*) with slight modifications and control measures as presented in the subsequent reports cited above. Recipient population densities generally ranged from 10^7 to 10^9 colony-forming units per ml and 40 μ g of DNA was employed per ml of the transformation mixture.

Continuous DNA exposure (without termination of DNA action by means of DNase) was used extensively. The semiquantitative application of this technique may have different purposes (*Boore 1964b 196a b c*). Because of its high sensitivity with a view to those transformation quantified in 15 min DNA exposure was principally as follows in experiments with highly competent recipients: the donor capacities of various heterologous organisms were compared with each other. If the activity of one or more of the donors happened to be just quantifiable in 15 min DNA exposure, continuous DNA exposure permitted rough translation into quantitative terms of parallel observations (see section of results and *Boore 1967*).

All parallels to be compared in the continuous DNA exposure experiments consisted of aliquots of the same recipient population simultaneously exposed to the same quantities of the various DNA extracts under identical conditions. The DNA preparations had been subjected to the same extraction process (*Boore 1964a*) and were measured for total DNA content by means of Dische's diphenylamine method. Recipient populations and DNA extracts were mixed in fluid transformation medium (*Boore 1964a*). Sometimes mixing was performed in an ice water bath and plating immediately thereafter. In other cases the mixtures were placed in the cold for plating after identical periods (15-30 min) at 3°-33° C. Usually 40 μ g of DNA was

at the same temperature and humidity before use.

The mutant DNA extracts were all prepared from the same mutant isolates as those used in previous investigations. The DNase sensitivity of the interactions to be reported was under control.

Transformation with DNA extracts prepared from first round transformants was performed by the usual quantitative streptomycin resistance transformation technique. Transformants employed as source of DNA had in general been selected at 50 μ g of streptomycin per ml but were all able to grow uniformly at 1000 μ g of streptomycin per ml and on media without streptomycin as shown by velvet replica plating and by ordinary surface streaking.

Transformation with haemolysis as the genetic marker was employed parallel to streptomycin resistance transformation in some instances using the same quantitative method with the exception that streptomycin addition was omitted in those parallels intended for assay of haemolytic transformants. The assay medium was human blood agar (*Boore 1964a*). As in the case of streptomycin resistant transformants the occurrence exclusively in surface cultures made assay and control of the haemolytic colonies or plaques easy. This was performed either by direct inspection of the plates studied or the blood agar after the colonies had been removed. Secondary spreading of suspected plaques or by combinations of these methods.

RESULTS

Streptomycin Resistance Transformation

In Table 1 are presented results of quantitative and semiquantitative transformation to streptomycin resistance of various competent positive

TABLE 1

Quantitative and Semiquantitative Streptomycin Resistance Transformation between Oxidase Positive Rods and Neisseriae with Rods as Recipients

Experiment no	Recipient strain	Donor strain	Ratio of inter to intrastain transformation in 15 min DNA exposure	Interstrain transformants per plate in continuous DNA exposure at μg per ml of streptomycin		
				10	50	500
1	5873	<i>V. flavescens</i> 13120	$<2.4 \times 10^{-6}$	0	0.0	0
		<i>V. catarrhalis</i> Ne11	$<2.4 \times 10^{-5}$	30	32.37	29
		<i>V. catarrhalis</i> 4103	$<2.4 \times 10^{-5}$	18	50.66	32
		<i>V. ovis</i> 199/55	$<2.4 \times 10^{-5}$	49	55.43	47
		<i>M. nonliq</i> 7784		39	44.51	45
		<i>M. nonliq</i> 4663/62		60	72.95	83
2	5873	<i>M. nonliq</i> 4663/62			81.100	
		<i>M. bovis</i> 10900			89.121	
		<i>M. liquefaciens</i> 9833	1.0×10^{-4}		314.232	
3	<i>M. nonliq</i> 7784	<i>V. flavescens</i> 13120	$<2.5 \times 10^{-5}$	0	0	0
		<i>V. catarrhalis</i> Ne11	$<2.5 \times 10^{-5}$	74	21	0
		<i>V. catarrhalis</i> 4103	$<2.5 \times 10^{-5}$	73	68	16
		<i>V. ovis</i> 199/55	$<2.5 \times 10^{-5}$	500*	500	21
		5873		16	15	3
		8134		29	27	8
		8292		16	16	7
		19116/51		18	18	2
4	<i>M. nonliq</i> 4663/62	<i>V. flavescens</i> 13120	$<2.9 \times 10^{-5}$	0	(4)4	0
		<i>V. catarrhalis</i> Ne11	$<2.9 \times 10^{-5}$	0	(1)8	0
		<i>V. catarrhalis</i> 4103	$<2.9 \times 10^{-5}$	41	55	3
		<i>V. ovis</i> 199/55	$<2.9 \times 10^{-5}$	600*	600	6
		5718		8	8	2
		5833		53	51	15
		9893		27	40	11
		10973		16	30	13
		4608		51	56	10
		A1920		31	30	28
5	<i>(M. lacunata)</i> A947(1)	<i>V. catarrhalis</i> Ne11	$<5.9 \times 10^{-6}$	0	0	
		<i>V. ovis</i> 199/55	2.9×10^{-6}	78	87.66	
		8292	$<5.9 \times 10^{-6}$	10	11.5	
6	<i>M. bovis</i> 10900	<i>N. envigae</i> 14659	$<1.6 \times 10^{-4}$	122	152	98
		<i>N. ovis</i> 199/55	$<1.6 \times 10^{-4}$	24	20	30
7	<i>M. bovis</i> 10900	<i>V. catarrhalis</i> Ne11		3	9	0
		<i>N. ovis</i> 199/55			225	234
		8292		16	18	17

N. = *Neisseria* *M.* = *Moraxella* *nonliq.* = *nontliquefaciens* Strains with no species designation belong to the 19116/51 group (Boire 1965 d) (*M. lacunata*) See Boire (1965 c) Experiment no refers to the semiquantitative part of the table (continuous DNA exposure) The latter results can be directly compared only within each experiment All single plate counts given The results of 15 min DNA exposure were obtained simultaneously with the semiquantitative results or in separate experiments See preceding communications for intrastain transformability and calculation of ratios $<$ = less than 1 interstrain transformant per 0.1 ml at uncritical streptomycin concentration * Approximate figures † Probably mutants

rods with DNAs of streptomycin resistant mutants of neisseriae and oxidase positive rods. The results of quantitative experiments may be supplemented with previously performed transformation between the 19116a1 group and other oxidase positive rods (Bovre 1964 b, 1965 a c d). In the semiquantitative tests transformants are noted in most of the combinations contrary to the results with 15 min DNA exposure. That some reactivity is observed between representatives of groups which previously have shown less striking or no interaction is not surprising in view of the following facts: (1) Slight differences in donor efficiency towards a given heterologous recipient may be seen within a group of closely interrelated donors. (2) Competence of a recipient strain may vary from time to time. (3) Recipients of the same group are not equally potent. (4) The sensitivity of the semiquantitative methodology has been improved.

Table 1 shows that with some recipients the yield of transformants is critically influenced by the highest selection concentration of streptomycin (500 μg per ml). After replication of transformant assay plates by the velvet method to plates with 500 and 1000 μg of streptomycin per ml practically all colonies assayed at low concentrations also grew secondarily at the high concentrations. Exceptional in this respect were approximately half of the transformants elicited at 10 μg by the *Neisseria calarrhalis* Ne 11 donor in experiment no. 3. Low yield at the 500 μg concentration may well reflect mechanisms shared by *Haemophilus* (Alexander & Leidy 1953). In the latter study low transformant counts at high selection concentration reached the higher level obtained at low concentration when the phenotypic expression period was prolonged. Fox (1959) concluded on the basis of studies in pneumococci that such findings almost entirely can be accounted for by a delay in action of streptomycin at low concentrations.

Indirect expression in quantitative terms of results in continuous DNA exposure may be illustrated by means of experiments no. 1 and 2 of Table 1. The average 323 transformants elicited by *Moraxella liquefaciens* in the '19116a1' group recipient of experiment no. 2 are equivalent to the ratio $4.0 \cdot 10^{-5}$ of inter- to intrastrain transformation. Then the 105 and 91 transformants elicited by *Moraxella bovis* and *Moraxella nonliquefaciens* in the same experiment are considered equivalent to a transformation compatibility of the order $1 \cdot 10^{-5}$. By extrapolating this value into experiment no. 1 the donor *Moraxella nonliquefaciens* 7784 reveals the approximate ratio $11 \cdot 10^{-6}$ with the '19116a1' group recipient.

Generally *Neisseria* and *Neisseria calarrhalis* 4103 reveal at least as high reactivity with the rods as the '19116a1' group shows with other oxidase positive rods (Table 1). This activity between rods and cocci seems particularly pronounced between the *Neisseria* as donor and the recipients recognized as unequivocal moraxellae. It is important to note here that *Neisseria catarrhalis* 4103 even gives reactivity to

TABLE 2

Quantitative and Semiquantitative Streptomycin Resistance Transformation between Oxidase Positive Rods and Neisseriae with Neisseriae as Recipients

Experiment no	Recipient strain	Donor strain	Ratio of inter to intrastain transformation in 15 min DNA exposure	Interstrain transformants per plate in continuous DNA exposure
1	<i>N. catarrhalis</i> Ne11	5873	< 9.9 × 10 ⁻⁷	64
		A1920		186
		8292		410
		<i>M. nonliq</i> 5050/62	4.0 × 10 ⁻⁵	
		<i>M. nonliq</i> 5058/62	3.8 × 10 ⁻⁵	
		<i>M. nonliq</i> 1333f/62	3.6 × 10 ⁻⁵	
		<i>M. nonliq</i> 7784	5.0 × 10 ⁻⁵	
			1.8 × 10 ⁻⁵	
		<i>M. nonliq</i> 4663/62	5.9 × 10 ⁻⁵	825
			7.4 × 10 ⁻⁵	
		<i>M. bovis</i> 10900	5.0 × 10 ⁻⁵	864
2	<i>N. catarrhalis</i> 4103	5873	< 1.5 × 10 ⁻⁵	406
		A1920		157
		8292		408
		<i>M. nonliq</i> 7784	< 1.5 × 10 ⁻⁵	
		<i>M. nonliq</i> 4663/62	< 1.5 × 10 ⁻⁵	194
3	<i>N. ovis</i> 199/55	<i>M. bovis</i> 10900	< 1.5 × 10 ⁻⁵	530
		<i>M. liquefaciens</i> 9833	< 1.5 × 10 ⁻⁵	570
3	<i>N. ovis</i> 199/55	5873	2.2 × 10 ⁻⁵	22
			3.1 × 10 ⁻⁵	
		A1920		233
		8292		613
		<i>M. nonliq</i> 7784	7.5 × 10 ⁻⁵	
			8.6 × 10 ⁻⁵	
		<i>M. nonliq</i> 4663/62	1.0 × 10 ⁻⁴	2200
			9.0 × 10 ⁻⁵	
		<i>M. bovis</i> 10900	6.6 × 10 ⁻⁵	10000
			6.2 × 10 ⁻⁵	
			8.4 × 10 ⁻⁵	
		<i>M. liquefaciens</i> 9833	1.1 × 10 ⁻⁴	5000
			2.4 × 10 ⁻⁴	
		<i>N. catarrhalis</i> Ne11	4.6 × 10 ⁻⁵	

Strain designations As in legend of Table 1

Experiment no refers to the semiquantitative part of the table (continuous DNA exposure). All figures based on at least two plate counts at 50 µg of streptomycin per ml (uncritical). The results of 15 min DNA exposure were obtained in separate experiments. See preceding communications for intrastain transformability and calculation of ratios < 1 = less than 1 transformant per 1 ml. * Approximate figures.

words the *Moraxella bovis* recipient than the *Neisseria ovis* donor. *Neisseria catarrhalis* Ne 11 shows irregular activity which may be a reflection of slightly lower resistance of transformants than usual. *Neisseria flavescens* is inactive in transformation of oxidase positive

rods, as it was towards *Neisseria catarrhalis* and *Neisseria ovis* recipients (Bovre 1965b)

In Table III are presented similar experiments with *Neisseria* recipients and oxidase positive rods as donors, in part compared with heterologous *Neisseria* donor activities (newly obtained values, for comparison see Bovre 1965b) The efficiency of transformation in this direction between rods and cocci appears greater than in the reverse reactions of Table I The reactions have been quantifiable in 15 min DNA exposure in most cases and the counts in continuous exposure are high In fact, the reactions of experiments similar to experiment no 1 of Table III were so high frequent that the sensitivity of continuous DNA exposure had to be geared down by using a dilute recipient population Thousands of colonies per plate were obtained in experiments not tabulated

Generally, the compatibilities of the *Neisseria catarrhalis* Ne 11 recipient with the oxidase positive rods are of the same order as between the same recipient and *Neisseria ovis* (Table 2) Between the *Neisseria ovis* recipient and the oxidase positive rods the affinities in these terms are partly much greater than between *Neisseria ovis* and *Neisseria catarrhalis* Particularly the reactions of *Neisseria ovis* with *Moraxella bovis* are of a relatively high order, with ratios of inter to intrastain transformation ranging from $6.2 \cdot 10^{-4}$ to $1.1 \cdot 10^{-3}$ Donors of the '1911651 group of oxidase positive rods appear least effective towards *Neisseria catarrhalis* Ne 11 (with indirectly calculated ratios down to $6 \cdot 10^{-6}$) and towards the *Neisseria ovis* recipient (with ratios down to $2.2 \cdot 10^{-5}$) The *Neisseria catarrhalis* 4103 recipient does not distinguish between the donors in the same manner and seems less receptive (for the deviation of strain 4103 from typical *Neisseria catarrhalis* strains, see Bovre 1965b) The relatively low donor efficiency of members of the '1911651 group on typical *Neisseria catarrhalis* organisms was also shown in a not tabulated experiment where *Neisseria catarrhalis* 8176 was transformed more efficiently by *Moraxella nonliquefaciens*, *Moraxella bovis* and *Moraxella liquefaciens* than by the '1911651 group donor 5893

Although irregularities are observed, the results of Table 2 generally point to consistency of relative affinities as determined in 15 min DNA exposure and continuous exposure This tendency particularly seen in experiment no II is considered of great importance for deductions from continuous exposure experiments

The interactions found between the *Neisseria ovis* recipient and *Moraxella bovis* are more frequent than those previously described between *Neisseria ovis* and *Neisseria caviae* (Bovre 1965b) The results presented in Table 3 where the effects on *Neisseria ovis* of three *Moraxella bovis* donors and two *Neisseria caviae* donors are compared in the same experimental setup, are corroborative on this point

From representative heterologous combinations listed in Tables I

TABLE 2

Quantitative and Semiquantitative Streptomycin Resistance Transformation between Oxidase Positive Rods and *Neisseriae* with *Neisseriae* as Recipients

Experiment no	Recipient strain	Donor strain	Ratio of inter to intrastrain transformation in 15 min DNA exposure	Interstrain transformants per plate in continuous DNA exposure
1	<i>N. catarrhalis</i> Ne11	5873	< 9.9 × 10 ⁻⁶	64
		41920		186
		8292	4.0 × 10 ⁻⁵	410
		<i>M. nonliq</i> 5050 62	3.8 × 10 ⁻⁵	
		<i>M. nonliq</i> 5053 62	3.8 × 10 ⁻⁵	
		<i>M. nonliq</i> 13536 62	3.5 × 10 ⁻⁵	
		<i>M. nonliq</i> 7784	5.0 × 10 ⁻⁵	
			1.8 × 10 ⁻⁵	
		<i>M. nonliq</i> 4663 62	5.9 × 10 ⁻⁵	825
			7.4 × 10 ⁻⁵	
		<i>M. bovis</i> 10900	5.0 × 10 ⁻⁵	462
2	<i>N. catarrhalis</i> 4103	5873	< 1.5 × 10 ⁻⁶	416
		41920		15
		8292		404
		<i>M. nonliq</i> 7784	< 1.5 × 10 ⁻⁵	
		<i>M. nonliq</i> 4663 62	< 1.5 × 10 ⁻⁵	194
		<i>M. bovis</i> 10900	< 1.5 × 10 ⁻⁵	531
		<i>M. liquefaciens</i> 9833	< 1.5 × 10 ⁻⁵	521
3	<i>N. ovis</i> 19935	5873	2.2 × 10 ⁻⁵	22
			3.1 × 10 ⁻⁵	
		41920		23
		8292	4.4 × 10 ⁻⁵	61
		<i>M. nonliq</i> 7784	7.5 × 10 ⁻⁵	
			8.6 × 10 ⁻⁵	
		<i>M. nonliq</i> 4663 62	1.0 × 10 ⁻⁴	2201
			9.0 × 10 ⁻⁵	
		<i>M. bovis</i> 10900	6.6 × 10 ⁻⁴	10001
			6.2 × 10 ⁻⁴	
			3.4 × 10 ⁻⁴	
		<i>M. liquefaciens</i> 9833	1.1 × 10 ⁻⁵	500
		<i>N. catarrhalis</i> Ne11	2.4 × 10 ⁻⁴	
			4.6 × 10 ⁻⁵	

Strain designations As in legend of Table 1

Experiment no refers to the semiquantitative part of the table (continuous DNA exposure) and figures based on at least two plate counts at 50 µg of streptomycin per ml (uncritical). The results of 15 min DNA exposure were obtained in separate experiments. See preceding communications for intrastrain transformability and calculation of ratios < = less than 1 transformant per 0.1 ml. * Approximate figures

wards the *Moraxella bovis* recipient than the *Neisseria ovis* donor. *Neisseria catarrhalis* Ne11 shows irregular activity which may be a reflection of slightly lower resistance of transformants than usual. *Neisseria flavescens* is inactive in transformation of oxidase positive

rods as it was towards *Veissaria calarrhalis* and *Veissaria ovis* recipients (Bovre 1963b)

In Table 2 are presented similar experiments with *Veissaria* recipients and oxidase positive rods as donors in part compared with heterologous *Veissaria* donor activities (newly obtained values for comparison see Bovre 1963b) The efficiency of transformation in this direction between rods and cocci appears greater than in the reverse reactions of Table 1 The reactions have been quantifiable in 15 min DNA exposure in most cases and the counts in continuous exposure are high In fact the reactions of experiments similar to experiment no 1 of Table 2 were so high frequent that the sensitivity of continuous DNA exposure had to be geared down by using a dilute recipient population Thousands of colonies per plate were obtained in experiments not tabulated

Generally the compatibilities of the *Veissaria calarrhalis* Ne 11 recipient with the oxidase positive rods are of the same order as between the same recipient and *Veissaria ovis* (Table 2) Between the *Veissaria ovis* recipient and the oxidase positive rods the affinities in these terms are partly much greater than between *Veissaria ovis* and *Veissaria calarrhalis* Particularly the reactions of *Veissaria ovis* with *Moraxella bovis* are of a relatively high order with ratios of inter- to intrastrain transformation ranging from $6.2 \cdot 10^{-4}$ to $1.1 \cdot 10^{-3}$ Donors of the 1911601 group of oxidase positive rods appear least effective towards *Veissaria calarrhalis* Ne 11 (with indirectly calculated ratios down to $6 \cdot 10^{-6}$) and towards the *Veissaria ovis* recipient (with ratios down to $2.2 \cdot 10^{-5}$) The *Veissaria calarrhalis* 4103 recipient does not distinguish between the donors in the same manner and seems less receptive (for the deviation of strain 4103 from typical *Veissaria calarrhalis* strains see Bovre 1963b) The relatively low donor efficiency of members of the "1911601" group on typical *Veissaria calarrhalis* organisms was also shown in a not tabulated experiment where *Veissaria calarrhalis* 8176 was transformed more efficiently by *Moraxella nonliquefaciens*, *Moraxella bovis* and *Moraxella liquefaciens* than by the 1911601 group donor 3893

Although irregularities are observed the results of Table 2 generally point to consistency of relative affinities as determined in 15 min DNA exposure and continuous exposure This tendency, particularly seen in experiment no 3 is considered of great importance for deductions from continuous exposure experiments

The interactions found between the *Veissaria ovis* recipient and *Moraxella bovis* are more frequent than those previously described between *Veissaria ovis* and *Veissaria catia* (Bovre 1961) The results of the experiments with the "1911601" group donors are in

TABLE 2

Quantitative and Semiquantitative Streptomycin Resistance Transformation between Oxidase Positive Rods and *Neisseriae* with *Neisseriae* as Recipients

Experiment no	Recipient strain	Donor strain	Ratio of inter to intrastrain transformation in 15 min DNA exposure	Interstrain transformants per plate in continuous DNA exposure
1	<i>N. catarrhalis</i> Ne11	5873	< 9.9 × 10 ⁻⁶	64
		A1920		186
		8292	4.0 × 10 ⁻⁶	410
		<i>M. nonliq</i> 5050/62	3.8 × 10 ⁻⁶	
		<i>M. nonliq</i> 5058/62	3.8 × 10 ⁻⁶	
		<i>M. nonliq</i> 13536/62	3.5 × 10 ⁻⁶	
		<i>M. nonliq</i> 7784	5.0 × 10 ⁻⁶	
			1.8 × 10 ⁻⁶	
		<i>M. nonliq</i> 4663/62	5.9 × 10 ⁻⁶	875
			7.4 × 10 ⁻⁶	
		<i>M. bovis</i> 10900	5.0 × 10 ⁻⁶	867
2	<i>N. catarrhalis</i> 4103		2.3 × 10 ⁻⁶	
		<i>M. liquefaciens</i> 9833	5.0 × 10 ⁻⁶	832
		<i>N. ovis</i> 19955	3.4 × 10 ⁻⁶	
		5873	< 1.5 × 10 ⁻⁶	406
		A1920		157
3	<i>N. ovis</i> 19955	8292		408
		<i>M. nonliq</i> 7784	< 1.5 × 10 ⁻⁶	
		<i>M. nonliq</i> 4663/62	< 1.5 × 10 ⁻⁶	194
		<i>M. bovis</i> 10900	< 1.5 × 10 ⁻⁶	530
		<i>M. liquefaciens</i> 9833	< 1.5 × 10 ⁻⁶	520
		5873	2.9 × 10 ⁻⁶	297
			3.1 × 10 ⁻⁶	
		A1920		233
		8292	4.4 × 10 ⁻⁶	613
		<i>M. nonliq</i> 7784	7.5 × 10 ⁻⁶	
			8.6 × 10 ⁻⁶	
		<i>M. nonliq</i> 4663/62	1.0 × 10 ⁻⁴	~200
			9.0 × 10 ⁻⁶	
		<i>M. bovis</i> 10900	6.6 × 10 ⁻⁴	10000*
			6.7 × 10 ⁻⁴	
			8.4 × 10 ⁻⁴	
			1.1 × 10 ⁻³	
		<i>M. liquefaciens</i> 9833	2.4 × 10 ⁻⁴	5000
		<i>N. catarrhalis</i> Ne11	4.6 × 10 ⁻⁶	

Strain designations: As in legend of Table 1

Experiment no refers to the semiquantitative part of the table (continuous DNA exposure). All figures based on at least two plate counts at 50 µg of streptomycin per ml (uncritical). The results of 15 min DNA exposure were obtained in separate experiments. See preceding communications for intrastrain transformation and calculation of ratios < 1 = less than 1 transformant per 0.1 ml. Approximate figures.

wards the *Moraxella bovis* recipient than the *Neisseria ovis* donor. *Neisseria catarrhalis* Ne11 shows irregular activity which may be a reflection of slightly lower resistance of transformants than usual. *Neisseria flavescens* is inactive in transformation of oxidase positive

TABLE 3
Streptomycin Resistance Transformation with DNAs Extracted from Transformants

Recipient strain	Donor strain	Origin of marker in donor strain*	DNA extract	Relative transformation frequency	
<i>V catarrhalis</i> Nell	5873	<i>V catarrhalis</i> Nell	(5873SmrNell)	no 1 no 1 no 3 no 1 no 1 no 3	59 10 ⁻² 89 10 ⁻² 12 10 ⁻¹ 15 10 ⁻² <29 10 ⁻⁵ 67 10 ⁻²
	<i>M nonlig</i> 7784		(7784SmrNell)		
	<i>V catarrhalis</i> Nell		(NellSmrNell)		1 (0.9-3.9%)§
<i>V catarrhalis</i> 4103	5873	<i>V catarrhalis</i> 4103	(5873Smr4103)	no 1 no 2	11 10 ⁻¹ 15 10 ⁻¹
	<i>M nonlig</i> 7784		(7784Smr4103)	no 1 no 2 no 3	11 10 ⁻³ 87 10 ⁻⁴ 13 10 ⁻³
	<i>N catarrhalis</i> 4103		(4103Smr4103)		1 (0.5%)§
<i>V ovis</i> 199/55	5873	<i>V ovis</i> 199/55	(5873Smr199/55)	no 1 no 1	26 10 ⁻² 24 10 ⁻²
	<i>M nonlig</i> 7784		(7784Smr199/55)	no 1 no 2 no 1	14 10 ⁻² 74 10 ⁻² 23 10 ⁻²
	<i>M bovis</i> 10900		(10900Smr199/55)	no 1 no 2 no 3	23 10 ⁻² 13 10 ⁻² 12 10 ⁻²
	<i>V ovis</i> 199/55		(199/55Smr199/55)		1 (0.5%)§

Legend identical with that of Table 4

TABLE 6
Streptomycin Resistance Transformation with DNAs Extracted from *Transformatis*

Recipient strain	Donor strain	Origin of marker in donor strain*	DNA extract	Relative transformation frequency
5873	5873	M nonlig 7784 Y <i>catarrhalis</i> Ne11 N <i>catarrhalis</i> 4103 V or is 19/55 5873	(5873Sm7784) no 3 (5873SmNe11) no 3 (5873Sm4103) no 2 (5873Sm19/55) no 1 (5873Sm5873)	11 100 75 101 12 100 79 101 1 (0.4 0.5%)
M nonlig 7784	M nonlig 7784	5873	(7784Sm5873) no 11	90 101
Y <i>catarrhalis</i> Ne11	Y nonlig 7784	5873	(7784SmNe11) no 3	65 101
N <i>catarrhalis</i> 4103	N <i>catarrhalis</i> 4103	5873	(7784Sm4103) no 3	10 100
V or is 19/55	V or is 19/55	5873	(7784Sm19/55) no 3	60 101
M nonlig 7784	M nonlig 7784	5873	(7784Sm7784)	1 (0.8%)
Y <i>catarrhalis</i> Ne11	Y <i>catarrhalis</i> Ne11	5873	(Ne11Sm5873) no 3	90 101
N <i>catarrhalis</i> 4103	N <i>catarrhalis</i> 4103	5873	(Ne11Sm7784) no 1	86 101
V or is 19/55	V or is 19/55	5873	(Ne11SmNe11)	97 101
M nonlig 7784	M nonlig 7784	5873	(Ne11SmNe11)	1 (1.2%)
Y <i>catarrhalis</i> Ne11	Y <i>catarrhalis</i> Ne11	5873	(4103Sm5873) no 2	74 101
N <i>catarrhalis</i> 4103	N <i>catarrhalis</i> 4103	5873	(4103Sm7784) no 3	11 101
V or is 19/55	V or is 19/55	5873	(4103Sm4103)	1 (1.0%)
M nonlig 7784	M nonlig 7784	5873	(19/55Sm5873) no 2	76 101
N <i>catarrhalis</i> 4103	N <i>catarrhalis</i> 4103	5873	(19/55Sm7784) no 1	92 101
V or is 19/55	V or is 19/55	5873	(19/55Sm10900)	96 101
M nonlig 7784	M nonlig 7784	5873	(19/55Sm19/55)	1 (1.4%)

Legend identical with that of Table 4

rologous factor) The results are presented in Table II The reactions are all of a very high efficiency (lowest value obtained $6.5 \cdot 10^{-1}$ of strictly homologous transformation frequency) Consequently, heterologies of the marker sites and adjacent parts of the genomes do not appear to have been of importance for reduction below a first degree compatibility (Bovre 1964b) of the primary transformation reactions between the actual microbes listed in Tables 1 and 2 Similar conclusions have been made on the basis of transformation within *Moraxella* (Bovre 1965a, c), within *Neisseria* (Bovre 1965b), and also in *Bacillus* (Marmur, Seaman & Levine 1963)

Plates from almost all positive experiments noted in Tables 2, 3, 4, 5 and 6 were replicated to plates with 1000 μg of streptomycin per ml With very few exceptions the transformant colonies grew at this concentration Exceptional were transformants with streptomycin resistance marker from *Neisseria catarrhalis* Ne 11, which in some instances tolerated only the next lower concentration tested (500 μg per ml)

First round transformants of all combinations used for DNA extraction were examined for eventual spontaneous loss of the transformed character after lyophilization and several subcultures in media devoid of streptomycin Approximately 50 000 single colonies were studied in simultaneous replica plating to media without streptomycin and supplied with 50 μg of streptomycin per ml No sign of instability was observed

Transformation of Haemolysis

In Figs 1 and 2 is presented the morphology of haemolytic zones on human blood agar produced by *Neisseria ovis* and *Moraxella bovis* colonies The figures are directly comparable

The first indication of genetic transfer of haemolysis from *Moraxella bovis* to *Neisseria ovis* was due to a chance observation on a plate with 10 000 heterologous streptomycin resistant transformants Several plaques of clear haemolysis could be seen on the background of diffuse but less penetrating *Neisseria ovis* haemolysis Secondary culture from the center of some of these plaques revealed *Neisseria ovis* colonies with accentuated haemolysis as shown in Fig 3 Subsequent attempts at exact determination of the transformation frequency were unsuccessful, because of the background haemolysis of the recipient

Fortunately, a completely nonhaemolytic mutant of *Neisseria ovis* 19935 was available again due to a chance observation at an earlier occasion This mutant was employed as recipient in the quantitative transformation experiments presented in Table 7 Donors were ordinary streptomycin resistant mutants of *Neisseria ovis* and *Moraxella bovis* The haemolysis of *Moraxella bovis* 9425 was as shown in Fig 2 Haemolytic transformants elicited by the *Neisseria ovis* donor were exclusively of the type shown in Fig 1, and those elicited by *Moraxella*

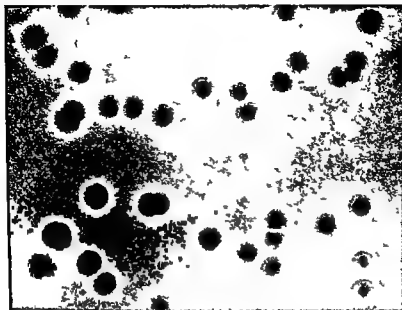


Fig 1
Neisseria meningitidis 199/93 haemolytic donor



Fig 2
Moraxella bovis x 10200 haem 1 1 n

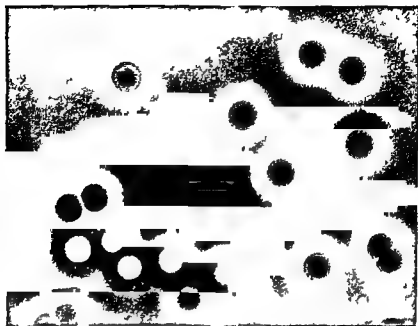


Fig 3

Haemolysis in *Neisseria ov*s 199/55 after transformation of its nonhaemolytic mutant with *Noraxella bovis* 10900 DNA

bovis donors were mainly of the type shown in Fig 3. However, approximately 10 per cent of the haemolytic transformants elicited by strain 10900 were of the Fig 1 type.

Streptomycin resistant and haemolytic transformants were assayed separately but in parallel under exactly comparable conditions. Double transformants were assayed on the plates with streptomycin addition.

It can be seen from Table 7 that the frequencies of streptomycin resistance and haemolysis transformation are of approximately the same order in homologous reactions. In heterologous reactions, however, the transformation with haemolysis as the genetic marker is much more efficient than with the streptomycin resistance marker.

The experiments of Table 7 show that 40 μ g of DNA per ml of the transformation mixture, which is now the routine concentration, is saturating with respect to both markers even with a very dense population of highly competent recipient bacteria. The relative number of double transformants is small and the decreasing proportion of double transformants with decreasing concentration of DNA below saturation further indicates that eventual linkage between the two markers in *Neisseria ov*s must be of low degree (see Goodgal 1961, Goodgal & Herriott 1961, Hayes 1964).

Homologous or heterologous DNA which had been treated with DNase beforehand never gave rise to haemolytic colonies. Occurrence of spontaneous haemolytic back mutants of the nonhaemolytic recipi-

ent was never observed. Some 11 000 widely separated transformant colonies did not reveal signs of spontaneous loss or other instability of the transformed haemolytic character.

TABLE 7

Transformation of a Nonhaemolytic Mutant of Streptomycin Sensitive Neisseria ovis 19955 to Haemolysis and Streptomycin Resistance with Doubly Marked Neisseria ovis and Moraxella bovis DNAs

Donor	Experiment no.	Recipient count/ml	μ g of DNA/ml	Streptomycin resistant transformants		Haemolytic transformants/ml
				Total count/ml	Per cent haemolytic clones	
<i>N. ovis</i> 19955	1	9.8 10^5	40	3.8 10^6	0.53	2.9 10^6
	2	4.8 10^5	40	1.9 10^6		1.6 10^6
	3	1.7 10^5	40	5.0 10^5		6.0 10^5
	4	9.3 10^5	80	5.4 10^5		8.8 10^5
			40	6.4 10^5		8.7 10^5
			20	5.9 10^5		8.2 10^5
			10	3.1 10^5		4.5 10^5
			1	9.6 10^5	0.16	
			1	5.3 10^5	0.13	
	5	2.3 10^5	0.02	2.0 10^5	0.04	
<i>M. bovis</i> 10900	6	5.0 10^5	40	5.2 10^5		1.3 10^5
	3	1.7 10^5	40	5.4 10^5		2.8 10^5
	4	2.3 10^5	80	4.7 10^5		3.3 10^5
			20	4.8 10^5		3.2 10^5
			10	5.0 10^5		3.1 10^5
			2	3.1 10^5		1.7 10^5
<i>M. bovis</i> 9425	7	8.7 10^5	40	1.1 10^5		1.0 10^5

15 min DNA exposure. Selection of streptomycin resistant transformants at 100 μ g of streptomycin per ml. Figures based on means of two plate counts. All observations within one experiment are simultaneous and directly comparable. Experiments no. 3 and 4 include both homologous and heterologous donors.

Attempts at transformation to haemolysis of two strains of *Moraxella nonliquefaciens* and one strain of *Moraxella liquefaciens* with *Moraxella bovis* DNA were unsuccessful in experiments where simultaneous streptomycin resistance transformation showed high activity. Transformation of *Neisseria catarrhalis* to haemolysis with *Neisseria ovis* DNA was also unsuccessful. On the other hand the weak haemolysis of *Neisseria caviae* seemed to be transferred at high frequency to the nonhaemolytic *Neisseria ovis* recipient. Assay of these transformants was very difficult, however, and exact values cannot be given.

DISCUSSION AND CONCLUSION

The relations in terms of streptomycin resistance transformation between *Neisseria catarrhalis* and oxidase positive rods previously re-

ported (Bovre 1963 Catlin 1964) are verified. The 19116:51 group seems to be situated at the lower part of the range of compatibility between oxidase positive rods and *Neisseria catarrhalis*. It must be noted however that assumed members of the 19116:51 group in the hands of Catlin (1964) were more active towards other *Neisseria catarrhalis* recipients than the strain No 11 used here. It is most valuable that Catlin in the same studies found time and DNA concentration response curves which are consistent with the transformation nature of the change to streptomycin resistance in these interactions.

There are some very low frequent interactions between the 19116:51 group and the other oxidase positive rods. These interactions are generally no more frequent than those observed when some neisseriae act as donors on rod recipients and are exceeded by the action of *Neisseria ovis* (and *Neisseria caviae*) on unequivocal *Moraxella* recipients.

The main outcome of this part of the studies is the relatively pronounced compatibility in streptomycin resistance transformation observed between *Neisseria ovis* and *Moraxella bovis*. These interactions are even more frequent than those found between *Neisseria ovis* and *Neisseria caviae* in one way experiments. The transformation of haemolysis at high frequency between *Moraxella bovis* and *Neisseria ovis* also indicates that there are particular relations between these organisms. Taxonomically important affinities thus seem to exist between *Moraxella* and *Neisseria* as suggested by Henriksen (1952). *Moraxella liquefaciens* and *Moraxella nonliquefaciens* are not very far removed from *Moraxella bovis* as regards transformation compatibility with *Neisseria ovis*. The 19116:51 group is apparently more distantly related to *Neisseria ovis* than the oxidase positive rods mentioned.

It is generally felt that the transformation reactions of the 19116:51 group with other oxidase positive organisms need further evaluation for eventual positive deductions on relationship. The same holds for the interactions between *Neisseria catarrhalis* and the oxidase positive rods in general although most of these compatibilities are comparable to that of *Neisseria catarrhalis* with *Neisseria ovis*.

As expected from its incompatibility with *Neisseria catarrhalis* *Neisseria flavescens* is incompatible in transformation also with the oxidase positive rods tested (see Bovre 1965b).

SUMMARY

On the basis of quantitative and semiquantitative streptomycin resistance transformation further evidence has been presented of genetic transfer between oxidase positive rods and asaccharolytic not yellow pigmented neisseriae.

No interaction exceeded those previously observed between *Morax*

ella nonliquefaciens, *Moraxella bovis* and the serum liquefying, non-haemolytic moraxellae

A typical *Neisseria catarrhalis* recipient was transformed with ratios of inter- to intrastain frequencies ranging from $1.8 \cdot 10^{-5}$ to $7.4 \cdot 10^{-4}$ by the moraxellae mentioned and with ratios ranging from $1 \cdot 10^{-6}$ to $4.0 \cdot 10^{-5}$ by members of the "1911651" group of oxidase positive rods

Neisseria ovis was transformed to streptomycin resistance by *Moraxella bovis* with the ratios $6.2 \cdot 10^{-4}$ to $1.1 \cdot 10^{-3}$ of inter- to intrastain transformation. Even higher compatibility was observed between the two in transformation with haemolysis as the genetic marker. The corresponding ratios ranged from $7.5 \cdot 10^{-5}$ to $1.4 \cdot 10^{-4}$ in reactions between the same recipient and donors of *Moraxella nonliquefaciens* and *Moraxella liquefaciens*. The "1911651" group was less reactive with *Neisseria ovis*.

Under the prevailing conditions, genetic transfer seemed less effective from cocci to rods than *vice versa*. Very low frequent interactions were observed between the "1911651" group and other oxidase positive rods. More frequent transformation was observed, however, when *Neisseria ovis* and *Neisseria caviae* acted as donors on unequivocal *Moraxella* recipients. *Neisseria catarrhalis* DNAs had some irregular activities on rod recipients whereas *Neisseria flavescens* was consistently negative.

The interaction between *Neisseria ovis* and *Moraxella bovis* are considered indicative of taxonomically important relations between *Moraxella* and *Neisseria*.

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STUDIES ON TRANSFORMATION IN *MORAXELLA* AND ORGANISMS ASSUMED TO BE RELATED TO *MORAXELLA*

8 The Relative Position of some Oxidase Negative, Immotile Diplo- bacilli (*Achromobacter*) in the Transformation System

By

KJELL BOVRE

Received 23 VI 66

Previous transformation experiments involving oxidase negative rods (Catlin & Cunningham 1964) have not clearly indicated reactivity with oxidase positive organisms. However, development of an extremely sensitive transformation method (Bovre 1967) has permitted supplementary investigations.

The affinities of oxidase negative, strictly aerobic and immotile diplobacilli are considered particularly important for a comparative evaluation of the low compatibilities found between the "19116.51" group and other oxidase positive organisms, and between *Neisseria catarrhalis* and oxidase positive rods (Bovre 1967).

MATERIAL AND METHODS

The oxidase negative immotile diplobacilli were represented by 10 strains of rather wide phenotypic diversity. In agreement with Dr. S. D. Henriksen all strains were provisionally called *Achromobacter*. The strains are listed in Table 1. The strains 8558/62, 742/56 and 2181/60 had been isolated from sputum and strain 17933 from urine (probably contaminant). The source of the remaining strains is unknown.

The *Achromobacter* strains were examined by conventional means as described (Bovre 1964b), with some modifications. Tests for growth in citrate media were extended to the use of Simmons Citrate Agar (Difco) in addition to the fluid and solid Hoser's medium previously used (Bovre 1965c). Gelatin liquefaction was substituted for serum liquefaction. The medium consisted of 15 per cent gelatin in nutrient broth. Acid production from glucose was tested for in Hugh & Leifson's medium (slab culture). The glucose oxidizing strains were also tested in 1 per cent peptone water with 1 per cent glucose, lactose, galactose, xylose, arabinose and 10 per cent lactose respectively. Methyl red was the indicator of acid production. The incubation periods for reading of gelatin liquefaction and acid production from carbohydrates are given in the legend of Table 1.

Various strains of the "19116.51" group were used in the present study. The following strains have been used in the transformation system:

TABLE 1
Designations and Origin of the *Achromobacter* Strains

Designation in this report	Other designations	Received from
<i>Achromobacter lwoffii</i> 17985	<i>Moraxella lwoffii</i> var. <i>bacteroides</i> (Institut Pasteur) <i>Moraxella lwoffii</i> var. <i>bacteroides</i> ATCC 17985	Institut Pasteur Paris
<i>Achromobacter lwoffii</i> 17987	<i>Moraxella lwoffii</i> var. <i>brevis</i> NCTC 5867 <i>Moraxella lwoffii</i> var. <i>brevis</i> ATCC 17987	NCTC
<i>Achromobacter lwoffii</i> 881/57		§
<i>Achromobacter lwoffii</i> 8858/62		§
<i>Achromobacter anitratus</i> 8	<i>Bacterium anitratum</i> 8 (SSI)	Dr H Lautrop, Copenhagen
<i>Achromobacter anitratus</i> 9	<i>Bacterium anitratum</i> 9 (SSI)	Dr H Lautrop, Copenhagen
<i>Achromobacter haemolysans</i> 742/56		§
<i>Achromobacter haemolysans</i> 2408/57		§
<i>Achromobacter haemolysans</i> 2181/60	<i>Achromobacter haemolyticus</i> var. <i>alcaligenes</i> 2181/60 (HIIH)	Dr W Mannheim Heidelberg
<i>Achromobacter haemolysans</i> 17988	<i>Alcaligenes haemolysans</i> ATCC 17988 <i>Alcaligenes haemolysans</i> 4233/62	§

ATCC = The American Type Culture Collection NCTC = The National Collection of Type Cultures London (SSI) = State Serum Institute Copenhagen (HIIH) = Hygiene-Institut d. Universität Heidelberg § Isolated in Kaptein W. Wilhelmsen (g. Frues Bakteriologiske Inst. Oslo)

ments *Neisseria meningitidis* M6 the unclassified *Pseudomonas* like strains 3806/56 and 9631/62 (all three isolated in this laboratory) *Alcaligenes faecalis* 415 1347 8769 and *Alcaligenes viscosus* 3233 (all four received from the National Collection of Type Cultures, London with the same designation). The wild type streptomycin sensitivity of the 7 strains was of the same order as that of *Achromobacter* strains (Table 3) and highly streptomycin resistant spontaneous mutants were used for DNA extraction. Some conventional characteristics of particular interest are noted in connection with their attempted use as donors of streptomycin resistance (Tables 4 and 6).

The methods of mutant selection and DNA extraction were in accordance with those described (Bøvre 1964a).

The continuous DNA exposure technique for comparison and approximate estimation of low transformation compatibilities (Bøvre 1967) was used in all experiments with mutant DNAs. Except for attempts at transfer of haemolysis (methodology described by Bøvre 1967) streptomycin resistance was the genetic marker. Although the mutants were highly resistant transformants were assayed at 50 µg of streptomycin per ml with controls at 10 µg per ml. Transformant numbers tabulated are means of two plate counts.

Three different experiments with the *Neisseria catarrhalis* Ne II recipient and the *Achromobacter lwoffii* 881/57 donor showed that maximum transformant yield was reached with 40 µg or smaller amounts of DNA per ml of the transformation mixture. Close to optimal concentration of DNA was therefore considered present in all ordinary experiments.

DNA spread on plates 4-5 h before the recipient bacteria had practically the same transforming effect as had the same amount spread simultaneously with the rec-

parallels to be compared were exposed to streptomycin at exactly the same time also this factor probably was of minor importance for proper deductions from the semiquantitative results. It must be emphasized however that different heterologous transformants may need different periods for phenotypic expression. The continuous exposure technique would not permit nondifferential assay of transformants in that case.

As in previous transformation studies it was shown that the interactions observed were prevented by DNase (in one case not tested see Results). It must also briefly be mentioned that the interactions were RNase insensitive (tested in several combinations but not all). Repeated DNA extractions and other control measures secured that the results reported were representative. Further methodological considerations are made under Results.

When transformant DNAs were employed for elucidation of eventual marker heterology the quantitative transformation technique with 15 min DNA exposure was used (Bovre 1964a).

RESULTS

Description of the Achromobacter Strains

Inconstancy of the microscopical picture of one and the same strain in different preparations was pronounced. Four of the strains are presented in Figs. 1-4. The remaining strains were mostly like Figs. 2 and 3 but as a rule with slightly smaller dimensions. Long filaments could be seen. All strains were Gram negative although many of them showed some resistance to decolorization.

The colonies had diameters ranging from 1.5 to 2.5 mm. They were generally of the low hemispherical or low conical type and were circular, even and glistening. The colonies of *Achromobacter lwoffii* had a rather moderate opacity with a clear periphery very little different from the 19116-01 group of oxidase positive rods (Bovre 1965d). The other strains had more opaque colonies. No pigmentation was observed on the various media used.

No difference was observed as regards growth ability at the two temperatures 32-33°C and 37°C. No strain was able to grow under completely anaerobic conditions (hydrogen atmosphere). Stab cultures in semisolid media revealed growth of all strains down to 5-10 mm below the surface.

No strain produced indol or hydrogen sulphide. Other characteristics of the strains are listed in Table 2. The haemolysis of *Achromobacter haemolyans* presented as wide completely clear zones (diameter 5-6 mm) after incubation for 20 h of human blood agar cultures. The haemolysis was bordered by a narrow zone of blood pigment accumulation which gave it a punched out appearance. *Achromobacter antraxus* 9 had a little smaller and less clearly delimited haemolytic zones. Growth on Hugh & Leifson's medium was fairly good of all strains on the surface as well as in stab culture. Direct comparison with the growth of the 19116-01 group revealed no clear cut difference. All *Achromobacter* strains except the two strains 17982 and 17987 were able to alkalize Simmons Citrate Agar. On strain 88157, however, at the same time seemed completely unable to grow in Koser's

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Some Characteristics of

Strain	Consistency of colonies	Agglutinability in physiological saline	Growth on Hugh & Leifson medium*
<i>A. lwoffii</i> 17985	Soft	—	+
<i>A. lwoffii</i> 17987	Mucoid adherent	—	+
<i>A. lwoffii</i> 881/57	Soft	—	+
<i>A. lwoffii</i> 8858/62	Soft	—	+
<i>A. anitratus</i> 8	Soft	—	+
<i>A. anitratus</i> 9	Soft	—	+
<i>A. haemolysans</i> 742/56	Soft	—	+
<i>A. haemolysans</i> 2408/57	Soft	—	+
<i>A. haemolysans</i> 2181/60	Soft	—	+
<i>A. haemolysans</i> 17988	Slightly friable	+	+

* See text for further description § — = Nitrate apparently not attacked as shown within 4 days // — = No acid production in 20 days on Hugh & Leifson's medium (1 per cent glucose galactose xylose arabinose and 10 per cent lactose respectively)

TAF
Antibiotic Sensitivity of the 10 *Achromobacter* Strains (Wild Type) Inhibi

Strain	Penicillin		Streptomycin	
	Zone in mm	Mic	Zone in mm	Mic
<i>A. lwoffii</i> 17985	22	14	24	0.6
<i>A. lwoffii</i> 17987	22	14	24	0.6
<i>A. lwoffii</i> 881/57	16	8	26	0.3
<i>A. lwoffii</i> 8858/62	<9	>50	30	0.05
<i>A. anitratus</i> 8	<9	>50	24	0.6
<i>A. anitratus</i> 9	9	>50	27	0
<i>A. haemolysans</i> 742/56	9	>50	26	0.3
<i>A. haemolysans</i> 2408/57	<9	>50	27	0.2
<i>A. haemolysans</i> 2181/60	14	13	24	0.6
<i>A. haemolysans</i> 17988	<9	>50	27	0.2

* Method of Frissson Högman & Wickman (1954) Mic values calculated from zone Mic values given as 1 U/ml for penicillin as µg/ml for the other antibiotics

fluid medium at 32–33° C and 37° C (only at one occasion) The strains 17985 and 17987 grew feebly on all the three citrate media Their growth was directly compared with that of the 19116/51 group and no clear cut difference was observed Although irregularities were observed no strain failed to grow in 1–2 days on more than one of the three media at the two incubation temperatures Difficulties in interpretation of weak growth on citrate media are liable to occur (see Broole 1951 Klinge 1959 and the section of Discussion and conclusion) It seems important to repeat the tests preferably on different media Extensive comparisons showed that the cases of weak growth in citrate reported in these studies represent a distinct property not

2 *Achromobacter Strains*

Growth on citrate media*	Nitrite production§	Urease activity	Haemolysis*	Gelatin liquefaction*	Acid production from carbohydrates
+	—	—	—	—	—
+	—	—	—	—	—
++	—	(+)+	—	—	—
++	—	—	—	—	—
++	—	—	—	—	+
++	—	—	+	+	+
++	—	—	+	+	+
++	—	—	+	+	+
++	—	—	+	+	—
++	—	—	+	+	—

means of Zn powder reduction * — = Negative after 20 days of incubation + = Positive (culture) + = Acid in 1-2 days on Hugh & Lefson's medium and in fluid media with acid in 20 days in fluid medium with 1 per cent lactose † Weakly positive in || 3 days

3 *Zone Diameters and Approximate Minimum Inhibitory Concentrations (m.i.c.)**

Chloromycetin		Oxytetracycline		Erythromycin	
Zone in mm	M.i.c.	Zone in mm	M.i.c.	Zone in mm	M.i.c.
30	0.6	24	0.6	27	0.8
29	0.8	25	0.4	24	2
30	0.6	24	0.6	25	1
30	0.6	22	1	24	2
19	9	23	0.9	20	6
11	>50	22	1	21	4
20	7	20	2	20	8
19	9	20	2	21	4
20	0.8	21	2	22	3
19	9	22	1	23	2

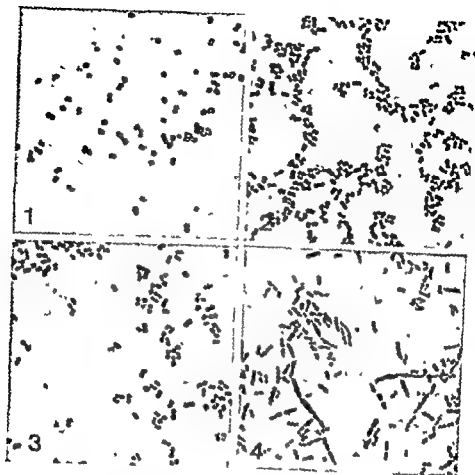
Diameters by means of regression equations for each antibiotic (Ericsson 1960)

shared by for instance the fastidious *Moraxella nonliquefaciens* (Dovre 1964b)

The sensitivity to antibiotics of the wild type organisms are presented in Table 3

Transformation Reactions

All strains of *Achromobacter* were tested for competence of being transformed in 15-30 min. exposure to own mutant DNA. All strains seemed incompetent. The strain *Achromobacter lwoffii* 88J36⁺ revealed a very slight transformability in continuous DNA exposure. Further transformation experiments with *Achromobacter* recipients were not undertaken.



Figs 1-4

Fig 1 *Achromobacter lwoffii* 17985Fig 3 *Achromobacter haemolysans* 17988Fig 2 *Achromobacter lwoffii* 881/57Fig 4 *Achromobacter haemolysans* 2408/57All stained by Gram's method $\times 1000$

Table 4 shows that strain 5873 of the "19116/51" group is transformed to streptomycin resistance by all the *Achromobacter* DNAs when the very sensitive continuous DNA exposure technique is employed. However, the transformant counts are very small and little impressive. The donors having the greatest similarity to the "19116/51" group in conventional tests (see above) show no peak of activity, and all *Achromobacter* donors are distinctly less effective than the *Moraxella nonliquefaciens* donors in the same experiments. The maximum ratio of inter- to intrastrain transformation between the "19116/51" group and *Achromobacter* can be roughly calculated to $1:10^6$ (using the activity of *Moraxella nonliquefaciens* 4663/62 as parameter; see Bövre 1967). Motile donors are inactive when applied in parallel.

The *Achromobacter* DNAs revealed no transformation compatibility

TABLE 4

Semiquantitative Streptomycin Resistance Transformation of Strain 5873 (of the 19116 51 Group Boire 1965 d) with Mutant DNAs of *Achromobacter* as Compared with the Effects of DNAs of *Moraxella nonliquefaciens* and some Motile Bacteria.

Experiment no	Donor strains	Resistant colonies per plate*	
		Total	Spontaneous mutants‡
1	<i>A lw</i> 831/57		1
	<i>A lw</i> 8353 6°		
	<i>A an</i> 9		
	<i>A haem</i> 742/56		
	<i>A haem</i> 2181/60	491	
	<i>M nonliq</i> 7784	246	
	<i>M nonliq</i> 4663/62	218	
2	<i>A an</i> 9	49	2
	<i>A haem</i> 742/56	47	
	<i>A haem</i> 2408/57	13	
	<i>A haem</i> 17983		
3	<i>A lw</i> 17983	30	3
	<i>M nonliq</i> 4663/62	690	
4	<i>A lw</i> 17987	29	2
	<i>A haem</i> 2408/57	53	
	<i>M nonliq</i> 7784	427	
	<i>M nonliq</i> 4626 6°	367	
	<i>M nonliq</i> 4663 6°	506	
	<i>M nonliq</i> 5050 6°	783	
	<i>M nonliq</i> 13536 6°	321	
	<i>Ps</i> 3806/56	2-4	
	<i>Alc</i> 415		
	<i>Alc</i> 3233		
	<i>Alc</i> 8769		

A lw = *Achromobacter lwoffii* *A an* = *Achromobacter anitralis* *A haem* = *Achromobacter haemolyticus* *M nonliq* = *Moraxella nonliquefaciens* *Ps* = *Pseudomonas* like donor (motile oxidase positive glucose-oxidizing) *Alc* = *Alcaligenes* donors (motile oxidase positive glucose negative)

* Figures can be directly compared only within each experiment

‡ Counted on parallel plates with DNase-degraded or no DNA

with another member of the 19116 51 group (strain A1920) *Moraxella nonliquefaciens* with activities of eventual *Achromobacter* compatibilities with the oxidase positive recipients. It is particularly interesting that the nitrate negative strain A1920 is incompatible with the *Achromobacter lwoffii* strains 17983 and 17987.

Table 6 shows the activities of all the *Achromobacter* DNAs towards the *Neisseria catarrhalis* Ne 11 recipient. The highest compatibility just exceeds the lowest observed between members of the 19116 51 group and the same recipient. However the *Achromobacter* activities are distinctly lower than those of *Moraxella nonliquefaciens*. The maximum ratio of inter- to intra-strain transformation between *Neisseria catarrhalis* Ne 11 and *Achromobacter* can indirectly be calculated to $7 \cdot 10^{-6}$ (using the activity of strain 8292 as parameter Boire 1967). Sensitive experiments were designed for comparison of the activity of *Achromobacter* donor with eventual activities of motile

TABLE 5
Attempts at Streptomycin Resistance Transformation of some Oxidase Positive Rods with DNAs of *Achromobacter*, with Positive Controls (Continuous DNA Exposure)

Experiment no	Recipient strain*	Active donor strain*	Resistant colonies per plate		Donors with no activity in same experiment*
			Total	Spontaneous mutants§	
1	A1920	M nonliq 4663/62	15	1	A lw 17985, A lw 17987, A lw 8858/62
2	M nonliq 7784	8292	35	1	A lw 881/57, A lw 8858/62, A haem 742/56 A haem 2408/57, A haem 2181/60, A haem 17988
3	M nonliq 7784	8292	75	3	A lw 17985, A lw 17987
4	M bovis 10900	ovis 199/55	175	2	A lw 881/57, A lw 8858/62, A an 9, A haem 2408/57, A haem 17988
5	M bovis 10900	8292 ovis 199/55	36 255	1	A lw 17985, A lw 17987
6	(M lacunata) A947(1)	8292 ovis 199/55	10 79	3	A lw 881/57, A lw 8858/62, A an 9, A haem 2408/57, A haem 17988

* M = *Moraxella nonliq* = *nonliquefaciens* V = *Veissaria* A lw = *Achromobacter lwoffii* A an = *Achromobacter anitratus*
A haem = *Achromobacter haemolyzans* Strains with no species designation belong to the 19116/51 group (Boire 1965 d)

§ Counted on parallel plates with DNase degraded or no DNA

TABLE 6

Semiquantitative Streptomycin Resistance Transformation of *Neisseria catarrhalis* Ne 11 with Mutant DNAs of *Achromobacter* as Compared with the Effects of Other Donors

Experiment no	Donor strains	Resistant colonies per plate*	
		Total	Spontaneous mutants†
1	<i>A lw</i> 881/57	56-286	4
	<i>A lw</i> 8858/62		
	<i>A an</i> 8		
	<i>A an</i> 9		
	<i>A haem</i> 747/56		
	<i>A haem</i> 2408/57		
2	<i>A haem</i> 2181/60	5000†	6
	<i>A haem</i> 17988		
	8292//		
	<i>M nonliq</i> 7784		
	<i>M nonliq</i> 1663/62		
3	<i>A lw</i> 17985	171 224	■
	<i>A lw</i> 17987		
	<i>A haem</i> 17988		
4	8292//	253	■
5	<i>A haem</i> 17988	457	■
	5873/		
	A1920//		
6		309	■
7	<i>A lw</i> 17987	352-436	7
	<i>A an</i> 8		
	<i>A haem</i> 17988		
8	<i>Ps</i> 3806/56	46	■
9	<i>A haem</i> 2181/60	135	8
	<i>Ps</i> 3806/56		
	<i>Ps</i> 9631/67		
10	<i>Ale</i> 415	■	■
	<i>Ale</i> 1347		
	<i>Ale</i> 3233		
11	<i>Ale</i> 8769	<10	■
	<i>N meningitidis</i> 116		
12	<i>A haem</i> 2181/60	167	8
	<i>N flavescens</i> 13120		

M nonliq = *Moraxella nonliquefaciens* *N* = *Neisseria* *A lw* = *Achromobacter lwoffii* *A an* = *Achromobacter anitratus* *A haem* = *Achromobacter haemolysans* *Ps* = *Pseudomonas* like donors (motile oxidase positive glucose-oxidase negative)

//

†

†

† Approximate figures

rods *Neisseria meningitidis* and *Neisseria flavescens*. Only one of the motile donors showed some activity (Table 6) which was reproducible with different DNA extracts.

Some irregularities inherent in the semiquantitative nature of the methodology are observed in Table 6 particularly as regards the relative activities of *Achromobacter haemolysans* 17988 and strain 8292 in different experiments.

The *Achromobacter* donor also had some effects on the recipients *Neisseria catarrhalis* 4103 and *Neisseria ovalis* which were much inferior to the lowest transforming effects of *Neisseria* recipients of oxidative positive rods and cocci. The point is that *Neisseria*

TABLE 7

Semiquantitative Streptomycin Resistance Transformation of *Neisseria catarrhalis* 4103 and *Neisseria ovis* 199/55 with Mutant DNAs of *Achromobacter*, Simultaneously Compared with Activities of Oxidase Positive, Immotile Donors

Experiment no	Recipient strain*	Donor strains*	Resistant colonies per plate	
			Total	Spontaneous mutants‡
1	<i>N. catarrhalis</i> 4103	<i>A. lw</i> 881/57 <i>A. lw</i> 8858/62 <i>A. an</i> 8 <i>A. an</i> 9 <i>A. haem</i> 742/56 <i>A. haem</i> 2408/57 <i>A. haem</i> 2181/60 <i>A. haem</i> 17988 8292	9.65 938	6
2	<i>N. catarrhalis</i> 4103	<i>A. lw</i> 17985 <i>A. lw</i> 17987 <i>A. lw</i> 881/57 8292	22-33 264	10
3	<i>N. ovis</i> 199/55	<i>A. lw</i> 881/57 <i>A. an</i> 8 <i>A. an</i> 9 <i>A. haem</i> 2181/60, <i>A. haem</i> 17988 <i>A. lw</i> 8858/62 <i>A. haem</i> 742/56 <i>A. haem</i> 2408/57 <i>M. nonliq</i> 7784 <i>M. nonliq</i> 4663/62	32.75 19 63 72 6500† 6500†	11
4	<i>N. ovis</i> 199/55	<i>A. lw</i> 17985 <i>A. lw</i> 17987 <i>A. haem</i> 2408/57	32.49 35	1
5	<i>N. ovis</i> 199/55	<i>A. haem</i> 742/56 5873 A1920 <i>N. catarrhalis</i> Dell	28 504 466 2800†	3
6	<i>N. ovis</i> 199/55	<i>A. lw</i> 8858/62 <i>N. flavescens</i> 13120	23 3	4

* *N.* = *Neisseria* *M.* = *Moraxella* *nonliq.* = *nontliquefaciens* *A. lw.* = *Achromobacter lwoffii*
A. an. = *Achromobacter anitratii* *A. haem.* = *Achromobacter haemolyans*

Strains with no species designation belong to the 19116/51 group (Bové 1965 d)

† Figures can be directly compared only within each experiment

‡ Counted on parallel plates without DNA † Approximate figures

flavescens donor, which again was inactive in an experiment where the least active *Achromobacter* donor showed activity (Table 7). The highest compatibility observed between the *Neisseria ovis* recipient and *Achromobacter* can be expressed by the ratio 11:10⁴ of inter- to intra-strain transformation (roughly estimated from the results of Table 7 using the activity of strain 5873 as parameter - see Bové 1967). It must be noted that the interactions between *Neisseria ovis* and *Achromobacter* were not controlled for DNase sensitivity. The activity was so low that the assumed transformation could not be reproduced regularly.

With the above mentioned exception, the results were generally

reproduced with a satisfactory exactness. In one puzzling case, however, distinctly deviating results were obtained with one particular DNA extract of *Achromobacter lwoffii* 17985. The extract had been stored at -20°C at the unusually low concentration of $75\text{ }\mu\text{g}$ per ml for 3 years before use and had an activity towards the recipient strain 5873 (of the "19116.51" group) which was 5–8 times as high as that of several extracts of *Moraxella nonliquefaciens* donors. Two freshly prepared extracts of the same mutant had the ordinary low effect on this recipient (Table 4). It may be argued that the higher heterologous activity observed was due to denaturation by storing at a low concentration. That the occurrence of heterologous transformants may depend on the presence of relatively short DNA fragments, has been suggested by Varmur, Seaman & Levine (1963). Own experiments with weak shearing forces (repeated vigorous expulsion through a narrow pipette opening) did not indicate that mechanical factors of the usual procedure could have been of importance in the heterologous reactions reported. Different degrees of purification are probably unimportant (Bovre 1964a).

In not tabulated quantitative experiments the strain 5873 and *Neisseria catarrhalis* Ne 11 were exposed to DNAs extracted from transformants elicited in these bacteria by *Achromobacter* donors. The only heterologous material of the donors in these second round transformation experiments thus was the part of the genome transferred with the marker in first round transformation. Ten transformant DNA extracts were involved and no one was less active than $4.5 \cdot 10^{-1}$ of completely homologous transformation. Provided that the picked colonies were transformants and not mutants, this result indicates close similarity of the nucleotide sequence of the marker sites in the organisms in question (see Bovre 1967).

Transformant assay plates from most of the combinations of first round transformation were replicated to $1000\text{ }\mu\text{g}$ of streptomycin per ml. With the exception of a couple of colonies on one occasion all transformants grew at this concentration. Some 14 000 of the first round transformants elicited by *Achromobacter* were tested for spontaneous loss of the acquired streptomycin resistance by replica plating to media without streptomycin and with $50\text{ }\mu\text{g}$ of streptomycin per ml. No instability was observed.

Attempts at transformation of haemolysis from *Achromobacter haemolysans* 17988 to *Neisseria catarrhalis* Ne 11 and from *Achromobacter anitralis* 9, *Achromobacter haemolysans* 74236 and *Achromobacter haemolysans* 17988 to a nonhaemolytic mutant of *Neisseria ovis* (see Bovre 1967), all failed.

DISCUSSION AND CONCLUSION

As a whole, the *Achromobacter* strains of the present material fulfil the criteria of *Actinobacter*, as proposed by *Brisou & Prevot* (1954) and *Brisou* (1957).

The strains 17985 and 17987 of *Achromobacter lwoffii* are in conventional tests similar to group 5 or *Achromobacter metalcaligenes* in the classification system of *Mannheim & Stenzel* (1962). The authors listed these organisms as citrate negative (not alkalizing), but it was noted that some of them were able to grow weakly on citrate. It seems that these two strains are similar to those *Moraxella lwoffii* strains examined by *Piechard, Piechard & Second* (1951) which grew on citrate media without alkalizing Simmons citrate. The other two *Achromobacter lwoffii* strains of the material seem to coincide with group 4 or *Achromobacter citroalcaligenes* of *Mannheim & Stenzel*. The remaining strains of *Achromobacter* included are presumably representatives of groups 1, 2 and 3 of the same system (*Achromobacter mucosus*, *Achromobacter haemolyticus* and possibly also *Achromobacter conjunctivae*). Since *Mima polymorpha* (*De Bord* 1939, 1942) and *Moraxella lwoffii* (*Audureau* 1940) probably are the same organisms (*Henriksen* 1963), the *Achromobacter lwoffii* strains of the material may be said also to represent *Mima polymorpha*. For further discussion of designations with relation to the material, *Henriksen* (1963), *Stenzel & Mannheim* (1963) and *Pickett & Wanclick* (1965) may be consulted.

It is evident from the study of conventional characteristics that the strains *Achromobacter lwoffii* 17985 and 17987 are virtually indistinguishable from nitrate negative members of the "19116/51" group of oxidase positive rods, except for the negative oxidase reaction. The genetic affinities of these organisms probably constitute main points on which to attack questions of relationship between oxidase positive and oxidase negative organisms.

Thus far, transformation experiments have given no proof or indication of close relationship between the "19116/51" group and oxidase negative rods. The very low frequent interactions observed cannot be overlooked, however, not least because motile donors tested in parallel were inefficient.

The importance of the interactions between the "19116/51" group donors and *Neisseria catarrhalis* may be doubted, since their frequencies are partly overlapped by *Achromobacter*/*Neisseria catarrhalis* interactions. However the absence of activity of other donors (most motile rods *Neisseria meningitidis* and *Neisseria flavescens*) throws some positive weight on the interactions of both combinations.

The "19116/51" group seems to be clearly closer to the unequivocal *Moraxellae* and *Neisseria ovis* than is *Achromobacter* in these terms. Generally the distinctly inferior transformation compatibilities of this

report as compared with those of the preceding one (Boure 1967) strengthen the belief that the interactions between the unequivocal moraxellae and *Neisseria ovis* have a significant taxonomic meaning. It is also shown that the interactions between the unequivocal moraxellae and *Neisseria catarrhalis* have a relatively outstanding efficiency.

SUMMARY

In semiquantitative streptomycin resistance transformation the activities of 10 *Achromobacter* donors were compared with the activities of selected oxidase positive donors towards various oxidase positive recipients. With few exceptions the *Achromobacter* donors were either inactive or exerted only a small fraction of the lowest efficiency shown by oxidase positive donors.

No indication was provided of close relationship between oxidase positive and oxidase negative rods; although some very infrequent interactions were observed between the 19116.01 group and *Achromobacter*. Between the latter and unequivocal moraxellae no transformation reactivity was found.

Interactions between *Achromobacter* donors and *Neisseria catarrhalis* were partly of the same efficiency as those found between the 19116.01 group and *Neisseria catarrhalis*. Towards a *Neisseria ovis* recipient *Achromobacter* was far less active than the least active donor of the 19116.51 group tested.

The donor capacity of *Achromobacter* was compared with donors of *Alcaligenes* and *Pseudomonas* like organisms towards recipients of the 19116.01 group and of *Neisseria catarrhalis*. With one exception the motile donors were inactive.

Interactions between nonpigmented asaccharolytic *Neisseria* recipients and unequivocal moraxellae were always more frequent than the reactions between the same recipients and *Achromobacter*. The notion that there are relations of taxonomic importance between *Neisseria* and *Moraxella* was therefore strengthened.

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TRANSFORMATION AND DNA BASE COMPOSITION
IN TAXONOMY, WITH
SPECIAL REFERENCE TO RECENT STUDIES IN
MORAXELLA AND *NEISSERIA*

By

KJELL BOVRE

Received 23 XI 66

The philosophy and outlines of new categories of approaches to bacterial taxonomy have recently been reviewed and discussed (Ravin 1960, 1963, Bisset 1962, Cowan 1962, 1965, Floodgate 1962, Roper 1962, Sneath 1962, 1964, Henriksen 1963a, Luria 1963, Marmur, Falkow & Mandel 1963, Pakula 1963, Skerman 1963, Sokal & Sneath 1963, Veron 1964 and Colobert 1965). One important theoretical direction is represented by the numerical analysis of phenotypic expressions. By this method all characteristics are given equal weight in the construction of classification groups. Another main trend is the use of genetic compatibility and homology of informational macromolecules (nucleic acids and proteins). Genetic transformation and overall DNA base ratio determination constitute important parts of the latter approach.

Although transformation and DNA base determination both have theoretical utility in taxonomy, their evaluation is to a large extent empirical. Previously obtained data are therefore of great importance for the subsequent discussion of own studies in *Moraxella*, *Neisseria* and some other organisms.

PREVIOUS APPLICATION
OF HETEROLOGOUS TRANSFORMATION

The present knowledge of transformation as a genetic phenomenon is mainly based on studies in *Haemophilus*, *Streptococcus*, *Bacillus* and *Neisseria* (see reviews by Ravin 1961, Marmur, Falkow & Mandel 1963, Hayes 1964, Schaeffer 1964). The majority of the studies concern intra-strain or autologous transformation. However, there are also illustrating examples of described activities between assumed species or types, which will be considered below.

General Principles

A feature reasonably common to "interspecific" transformation reactions is their low frequency when compared with the "intraspecific" kind. However, in some instances reactions exceeding parallel intra-strain reactivity have been observed (Callin & Cunningham 1961, Marmur, Seaman & Levine 1963). Such recordings might have been due to slightly different markers in the two parallels, so that transformant selection has been differential.

The ratio of inter- to intrastrain transformation is generally considered to be independent of such variables as DNA concentration, DNA exposure time and time allowed for phenotypic expression (Schaeffer 1958). No case of infrequent (but positive) intertransformation has been shown to be due to less effective penetration of heterologous than homologous DNA. Nondifferential penetration of DNA generally seems to have a wider distribution than the intertransformation phenomenon (see Boure 1964 and Schaeffer 1964 for discussion and references).

According to the imperfect-pairing hypothesis of Schaeffer (1958) the lower inter- than intrastrain transformation frequencies are due to incomplete homologies of the genomes in question, with resulting imperfect pairing of recipient and donor DNAs. As also emphasized by Schaeffer (1964) the imperfect pairing hypothesis would be complicated by the eventual presence in transformation of mechanisms similar to host-induced variation of bacteriophage and of bacterial DNA in transduction, conjugation and episomal transfer (Arber & Dussoix 1962, Dussoix & Arber 1962, Arber 1964, Arber & Horse 1965, Wood 1966). The operation of such factors would not necessarily invalidate transformation as a measure of relatedness, however.

Transformation in *Haemophilus*

Schaeffer & Ritz (1955) and Alexander & Leidy (1955) provided for the first time evidence of transformation between species of *Haemophilus*. Leidy, Hahn & Alexander (1956) reported ratios of inter- to intrastrain streptomycin resistance transformation ranging from $2 \cdot 10^{-7}$ to $2 \cdot 10^{-9}$ (in the present author's own terminology) between *Haemophilus influenzae* and *Haemophilus parainfluenzae*. Between the former and *Haemophilus suis* (not requiring X factor, however) the ratios ranged from $1 \cdot 10^{-4}$ to $4 \cdot 10^{-4}$. These values were verified with a very striking accuracy by Schaeffer (1956, 1958). Leidy, Hahn & Alexander (1959) found corresponding ratios ranging from $1/5$ to 1 between *Haemophilus influenzae* and *Haemophilus aegyptius*.

On the basis of their transformation results Leidy, Hahn & Alexander (1956) concluded that *Haemophilus influenzae* is more closely related to *Haemophilus parainfluenzae* than to the strain called *Haemophilus suis*. Further evidence of genetic homology between *Haemophilus*

influenzae and *Haemophilus aegyptius* was presented by Leidy, Jaffee & Alexander (1960). *Haemophilus influenzae* and *Haemophilus aegyptius* are morphologically very similar and it may be questioned even on the basis of conventional criteria whether they deserve species distinction.

The transformation compatibilities of *Haemophilus influenzae*, *Haemophilus parainfluenzae* and the type of *Haemophilus suis* used above are reflected in the guanine + cytosine (G + C) contents of their DNAs: 38, 39 and 41 per cent respectively (Schaeffer, Edgar & Rolfe 1960). Marmur, Falkow & Mandel (review 1963) reported G + C values from 38 to 40 per cent of all the *Haemophilus* species mentioned including *Haemophilus aegyptius*.

Transformation in Streptococceae

Bracco *et al.* (1957), Pakula, Fluder, Hulanicka & Walczak (1958) and Pakula, Hulanicka & Walczak (1958, 1959) reported transformation reactions between species of *Streptococcus* and partly also between *Diplococcus pneumoniae* and streptococci. Quantitative results were given by Pakula (1961, 1963). Calculations from his figures show ratios of inter- to intrastrain streptomycin resistance transformation of the order 1 between *Streptococcus sanguis* and a strain of group H streptococci in reciprocal tests. Between these strains and *Diplococcus pneumoniae* ratios ranged from $8 \cdot 10^{-3}$ to $6 \cdot 10^{-4}$, also in reciprocal tests. With *Streptococcus mitis* donors the *Diplococcus pneumoniae* recipient revealed ratios from $2 \cdot 10^{-3}$ to $1 \cdot 10^{-1}$. Between the same recipient and donors of *Streptococcus salivarius* group A and group C streptococci ratios of the order $4 \cdot 10^{-4}$ to $9 \cdot 10^{-3}$ were found. Between the group H and *Streptococcus sanguis* recipients and donors of *Streptococcus mitis* ratios were of the order $1 \cdot 10^{-4}$ to $1 \cdot 10^{-2}$ and between the same recipients and *Streptococcus salivarius*, group A and group C donors ratios ranged from $2 \cdot 10^{-4}$ to $9 \cdot 10^{-3}$. With a *Streptococcus faecalis* donor the ratios ranged from $4 \cdot 10^{-4}$ to $8 \cdot 10^{-4}$.

Results of streptomycin resistance transformation performed by Perry & Slade (1962, 1964) in *Streptococcus* were in close agreement with the studies of Pakula and collaborators. Further evidence of genetic homology between group H streptococci and *Diplococcus pneumoniae* was provided by Ratin (1963) and Rabin & De Sa (1964).

On the basis of his own results Pakula (1963) concluded that *Diplococcus pneumoniae* is closer in these terms to some streptococci than many streptococci are to each other and emphasized the agreement with the classification of Torrey and Wilson (Wilson & Miles 1955 and also 1964) where pneumococci and streptococci are not separated as distinct genera. He also emphasized that the genetic evidence of species identity of *Streptococcus sanguis* and group H streptococci corroborates physiological and immunological data. The results with

Streptococcus faecalis DNA were taken to indicate its also otherwise presumed distant relationship to other streptococci

Overall DNA base ratios of *Diplococcus pneumoniae* *Streptococcus salivarius*, *Streptococcus sanguis* *Streptococcus viridans* and *Streptococcus pyogenes* are all situated at 38-40 per cent G + C *Streptococcus faecalis* has G + C values in the interval 34-36 per cent (Marmur, Falow & Mandel 1963)

Transformation in *Bacillus*

Dubnau et al (1965) transformed *Bacillus subtilis* (43 per cent G + C) to streptomycin resistance with DNAs of *Bacillus niger* (43 per cent G + C) *Bacillus pumilis* (40-41 per cent G + C) and *Bacillus licheniformis* (45-46 per cent G + C) The ratios of inter- to intra-strain transformation were $5.4 \cdot 10^{-3}$ $2.3 \cdot 10^{-2}$ and $4 \cdot 10^{-3}$ respectively Transformation with biochemical markers was much inferior or negative in the same study, which is in accordance with previously obtained results in *Bacillus* (Marmur, Seaman & Levine 1961)

Transformation in *Neisseria*

Callin (1960-1961) and Callin & Cunningham (1961) examined *Neisseria meningitidis* *Neisseria flava* *Neisseria perflava* *Neisseria subflava* *Neisseria sicca* and *Neisseria flavescens* in qualitative streptomycin resistance transformation Between all these organisms ratios of inter- to intra-strain transformation were $1 \cdot 10^{-2}$ or higher Between *Neisseria flava* *Neisseria perflava* and *Neisseria subflava* ratios were higher than 1/10 (in both directions and all possible combinations) On the other hand a highly competent *Neisseria catarrhalis* recipient underwent virtually no transformation in response to DNAs from the above mentioned organisms (ratios less than $2 \cdot 10^{-6}$) In striking correlation with this were results of DNA base determinations (Callin & Cunningham 1961) which revealed approximately 41 per cent G + C for *Neisseria catarrhalis* and approximately 50 per cent G + C for the other organisms The authors stated that the inclusion of *catarrhalis* strains in genus *Neisseria* appears illogical from the evolutionary point of view whereas the other six organisms mentioned might be regarded as members of a single group (group I) The authors also emphasized that the combination of *Neisseria flava* *Neisseria perflava* and *Neisseria subflava* into a single species is suggested by Wilson & Miles (1955 and also 1964) is in harmony with evidence from the transformation experiments

DNA BASE COMPOSITION AND ITS REACTION TO TAXONOMY

Among bacteria the overall base composition of DNA as expressed in mole per cent guanine + cytosine (G + C) of total bases varies from 25 to 75 approximately (Saeola 1961) Within a bacterial strain the

G + C content shows a unimodal distribution and the range of heterogeneity between DNA molecules is relatively narrow (Rolfe & Meselson 1959, Sueoka 1961). Within molecules, however, the range of local composition seems rather broad (Guild 1963), contrary to earlier belief (Sueoka 1961).

Generally, similar overall DNA base composition is considered a necessary requirement for extensive base sequence homology, as reflected in genetic compatibility and integration (Lanni 1960, Luria 1963, Marmur, Falkow & Mandel 1963). However, transformation may occur between microbes which differ in G + C content by as much as 3-4 per cent (Callin 1964, Callin & Cunningham 1964a, Dubnau *et al* 1965). Some instances of genetic transfer between organisms of widely different G + C contents have been reviewed by Marmur, Falkow & Mandel (1963). In these cases the genetic information is considered to be mediated by episomes which are not integrated in the resident chromosome. Falkow *et al* (1961) and Marmur *et al* (1961) have presented very clear evidence of such transfer between *Salmonella typhosa* and *Escherichia coli* (both 50 per cent G + C) on one side and *Serratia marcescens* (58 per cent G + C) on the other. However, there probably also exists a limit to the range of base composition compatible with episomal infection. It has been suggested that this mode of genetic transfer is indicative of large taxonomic groupings, such as a family (Marmur, Falkow & Mandel 1963, Mandel 1966).

On the other hand, identical or closely similar DNA base ratios are evidently not sufficient for genetic interaction to occur, as emphasized for transformation in *Bacillus* (Marmur, Seaman & Levine 1963). Other illustrating examples are some combinations of enterobacteria, in which inferior efficiency in transduction and conjugation reflects only partial base sequence homology among organisms of similar overall DNA base composition (Schildkraut, Marmur & Doty 1961, Marmur, Falkow & Mandel 1963).

A large body of information has been made available on overall DNA base composition of bacteria since Lee, Wahl & Barbu (1956) presented the first analysis of its importance as a taxonomic aid. Reports (the first four relatively extensive reviews) which together contain most of the data known are those of Sueoka (1961), Marmur & Doty (1962), Schildkraut, Marmur & Doty (1962), Marmur, Falkow & Mandel (1963), Callin & Cunningham (1961, 1964a, b), Sebald & Veron (1963), Colwell & Mandel (1964), De Ley & Rassel (1965), Mandel & Leadbetter (1965), Seese, Jeffries & Mandel (1965), De Ley *et al* (1966), Gasser & Sebald (1966), LaMacchia & Pelczar (1966) and Mandel (1966).

In some cases good correlation has been found between overall DNA base composition and groups constructed by means of numerical analysis (Colwell & Mandel 1964, Colwell, Mandel & Gochmayer 1964, De Ley & Rassel 1965, Silvestri & Hill 1965). The majority of species

presently organized in particular genera seem to have DNA base compositions of quite similar order, and differences in these terms are often clear cut between higher taxa of classical taxonomy. There are exceptions, however. In some cases heterogeneity is so great within a taxon that it calls for reconsideration of the classification system. In other cases identity or close similarity in DNA base composition is found among microbes of great phenotypic diversity, as between *Streptococcae*, *Proteus vulgaris*, *Haemophilus influenzae*, *Bacillus megaterium* and *Achromobacter* strains. Determination of overall DNA base composition therefore has its limitations in taxonomy when used alone.

ON STUDIES IN *NEisseria* *NEisseria* AND SOME OTHER ORGANISMS

The transformation studies to be commented upon have been published elsewhere (Bovre 1964 a, b, 1965 a, b, c, d, 1967 a, b). The strains employed and the main results are listed in Tables 1, 3 and 4.

Results of DNA base determinations, performed by Dr W. Szybalski* with the CsCl buoyant density method on DNA extracts prepared by the author, are given in Tables 2 and 3. The results were generally not available before the respective transformation experiments had been finished. The methodology of the DNA base determinations will be included in a separate report (Bovre, Szybalski & Flandt 1966).

The Marker System and its Implications

The streptomycin system seems to be essentially similar in the strains involved, at least as regards interacting strains. A satisfactory marker uniformity is indicated by high degrees of sensitivity of all wild type organisms towards this antibiotic, by high degree of streptomycin resistance in all spontaneous one step mutants used as donors and by wide distribution of transformation with this marker.

Numerous experiments with integration of marker of heterologous origin in various donors have revealed no marker associated heterologies capable of reducing a first degree compatibility (whenever the term marker is used in this section, streptomycin resistance is meant if not otherwise stated). The incompatibility of *Neisseria cinerea* and *Neisseria flavescens* with the other oxidase positive organisms is paralleled by obvious gaps in terms of G + C content (Table 2), and the importance of discussion of marker associated heterologies is reduced in these cases.

It has been shown that heterologous transformation reactions are positively influenced by previous integration (in transformation) of homologous marker in the heterologous donor. In that manner very low frequent first round transformation has been raised to an inter-

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mediate or higher level in second round transformation. It may be suggested that homology restricted to the marker site can allow for only extremely low frequent transformation if any. To what extent more extensive but still particularly marker associated homologies may affect transformation frequency in a positive way without artificial preintegration is undecided. However, the most low frequent reactions will be basis of very limited taxonomic conclusions. In transformation of intermediate efficiency DNA base composition and transformation results with another marker (haemolysis) indicate widespread homologies (see below).

Some of the points emphasized above are based on the assumption that Schaeffer's pairing hypothesis is valid (see previous discussion) and not complicated by differential expression of intrastrain and interstrain transformants. It has repeatedly been observed that mutants at the streptomycin locus may have a pleiotropic nature with one or more metabolic requirements (Gorini & Beckwith 1966 review). In *R. obum*, nutritionally restricted streptomycin resistant mutants were observed (R. Balassa 1954 G. Lalassa 1963). It appeared that the development of heterologous transformants depended on the same factors as those required for development of mutants of the donors in question (not the recipients). It is therefore important to note that in the present studies the donor mutants were selected in the same manner and on the same media as was the case with transformants.

Although streptomycin resistance transformation may be differentially depressed even between closely related strains the deviations observed in these cases have been relatively small (Green 1959, Rolheim 1962). Lowering of the transformant selection concentration of streptomycin may be of importance (Bryan 1961, Pakula & Hulanicka-Banikowska 1961, Rolheim & Ravin 1961, Callin & Cunningham 1964, Boure 1965, 1967) eventually uncovering what might be called true transformation frequency.

Mutations at the streptomycin locus correspond to structural changes in ribosomes (Spotts & Slater 1961, Cox, White & Illaks 1961, Davies 1964). Ribosomes play an important part in the process of translation of the genetic message and streptomycin is considered to exert its effect by interfering with translation (Gorini & Kalaja 1961, Davies, Gilbert & Gorini 1964, Gorini & Beckwith 1966 review). It may therefore be argued that the streptomycin system has intimate relation to a widespread function so that integration and expression barriers are less likely to occur with streptomycin resistance markers as compared with some other markers. Rabin & De Sa (1964) emphasized the widespread homology of the streptomycin locus among the streptococci, pneumococci and stated that mutual recognition of this locus persists between species and as ability to transform each other declines. Dulman et al. (1967) presented results of interspecific transformation in *Bacillus* which support the existence of a core of stable genetic

presently organized in particular genera seem to have DNA base compositions of quite similar order and differences in these terms are often clear cut between higher taxa of classical taxonomy. There are exceptions however. In some cases heterogeneity is so great within a taxon that it calls for reconsideration of the classification system. In other cases identity or close similarity in DNA base composition is found among microbes of great phenotypic diversity, as between *Streptococcae*, *Proteus vulgaris*, *Haemophilus influenzae*, *Bacillus megaterium* and *Achromobacter* strains. Determination of overall DNA base composition therefore has its limitations in taxonomy when used alone.

OWN STUDIES IN *MORAXELLA* *NEISSERIA* AND SOME OTHER ORGANISMS

The transformation studies to be commented upon have been published elsewhere (Bovre 1964 a b 1965 a b, c d 1967 a b). The strains employed and the main results are listed in Tables 1, 2 and 4.

Results of DNA base determinations, performed by Dr W. Szybalski* with the CsCl buoyant density method on DNA extracts prepared by the author are given in Tables 2 and 3. The results were generally not available before the respective transformation experiments had been finished. The methodology of the DNA base determinations will be included in a separate report (Bovre, Szybalski & Flandt 1966).

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It has been shown that heterologous transformation reactions are positively influenced by previous integration (in transformation) of homologous marker in the heterologous donor. In that manner very low frequent first round transformation has been raised to an inter-

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mediate or higher level in second round transformation. It may be suggested that homology restricted to the marker site can allow for only extremely low frequent transformation if any. To what extent more extensive but still particularly marker associated homologies may affect transformation frequency in a positive way without artificial preintegration is undecided. However the most low frequent reactions will be basis of very limited taxonomic conclusions. In transformation of intermediate efficiency DNA base composition and transformation results with another marker (haemolysis) indicate widespread homologies (see below).

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material, relatively resistant to evolutionary change. Included in this core is the streptomycin locus. Ratios of inter- to intrastrain transformation with several biochemical markers were below 5-10, even between strains of the same species, whereas corresponding ratios with the streptomycin resistance marker kept easily measurable between species, falling off gradually with deviation of overall DNA base composition.

In contrast to the apparently uniform distribution of genetic fundamentals and phenotypic expression of streptomycin resistance, stands the transformation of haemolysis (Bovre 1967 a, b). This natural marker is evidently not of the ubiquitary character wanted for the purpose of the present studies.

Ravin (1963) suggested that some instances of interspecific transformation in the past may have been due to infection by an unintegrated episome (for reviews on episomes, see Jacob, Schaeffer & Wollman 1960, Campbell 1962, Driskell-Zamenhof 1964, Hayes 1964). Discussion of such a mechanism in the present system does not seem relevant, however, since the DNA extraction procedure employed (Bovre 1964 a) with great probability is incompatible with persistence of an eventual infectious episome (Jysum 1966). The unknown genetic determinant of haemolysis might nevertheless have an extrachromosomal location although the stability of the transferred character seemed great.

General Deductions from the Transformation Data

Streptomycin resistance transformation appears to be a valuable aid in exact ordering and control of identity of individual strains. A few examples will be mentioned.

It now seems that *Neisseria catarrhalis* usually is nitrate reducing (Bovre 1965 b). Nitrate negative, asaccharolytic neisseriae are not easily classified (*Neisseria cinerea* and *Neisseria catarrhalis* 4103, see below). The nitrate negative strain *Neisseria catarrhalis* 13074/62 (Bovre 1965 b) was found to possess a first degree relationship in terms of streptomycin resistance transformation to nitrate positive *Neisseria catarrhalis* organisms. It was consequently proposed that the strain was an unusual variety of *Neisseria catarrhalis*, but not representing a distinct taxonomic entity. DNA base ratios available at a later stage corroborated the transformation result (Tables 1 and 2).

During the studies a nonhaemolytic variety of *Neisseria ovis* was isolated. A nonhaemolytic *Neisseria ovis* is practically indistinguishable from *Neisseria catarrhalis* in the conventional tests used (Bovre 1965 b). Quantitative transformation with two markers revealed the true nature of the variety (Bovre 1967 a).

The strains 19116/51 and 752/52 both deviated from other assumed *Moraxella nonliquefaciens* strains in conventional tests (Bovre 1964 b). In transformation the deviation was very distinct and once more the DNA base ratios were corroborative (Tables 1 and 2).

TABLE 1
Strains Studied by means of Streptomycin Resistance Transformation Ratios of Inter- to Intrastrain Transformation

Proximal species or group designation*	Strain designations (I)	(II)	Ratios of Inter- to Intrastrain transformation	
			Among (I)	Between (I) and (II)
<i>Moraxella nonliquifaciens</i>	7781 872/58, 270 C0 2770/10, 3838 C0, 826/61, 836/61, 159/62		34 10 1	
	178 62, 1962/62, 423/62, 4378/62, 4626 62, 4661 62, 4861/62, 5050/62		-9 9 10 1	
	752/52			<21 10 8
	19116/518			<21 10 8
	17636/62			
<i>Moraxella bovis</i>	8561, 9425, 9426, 10900		53 10 1	
			-11 100	
<i>Serratia liquefaciens</i> <i>nonhaemolytic moraxellae</i>	7911, 9633 9985, 111, 11, 1047(1), 11748		10 10 1	
			-9 6 10 1	
	19116/51* group of all have positive rods		12 10 1	
<i>Serratia marcescens</i>	10973, 19116/61, 1708 11920		-10 100	
	Nell, 8176 12910/62 13016/62, 17074/62 13135/62, 13430 62		30 10 1	
	2817/63, 2982/61, 2993/61, 7889/63		-9 4 10 1	
	1165/64 1179/64 2124/63	4103		54 10 4 -4 0 10 3
<i>Yersinia enterocolitica</i>	10291, 14659		Incompetent†	
<i>Yersinia enterocolitica</i>	199/51, 57/59, 917/60		91 10 1	
			-9 5 10 1	
<i>Yersinia enterocolitica</i>	165/61, 159 62		Incompetent†	
<i>Yersinia enterocolitica</i>	8283, 13115 13120		4 10 1*	
<i>Achromobacter</i>	17985, 17987, 881/57, 8858 62, 8 9 742/56 2108/57, 2181/60 17988		Incompetent†	

* The conventional characteristics of all strains have been reported (Börre 1961b, 1965 a, b, c, d, 1967 b) † Representative of the "19116/51" group ‡ No transformants in 15 min exposure to own mutant DNA § Semiquantitative estimate. ¶ See Table 2

TABLE 2

Mean Guanine + Cytosine (G + C) Content of DNA Extracted from Oxidase Positive and Oxidase Negative Organisms (Part of the Strains of Table 1).

Provisional species or group designation	Strains examined	CsCl buoyant density (g/cm ³)	Mole per cent G + C
<i>Moraxella nonliquefaciens</i>	7784, 2770/60, 3828/60, 826/61, 836/61, 4378/62, 4863/62, 5058/62, 13536/62 178/62 5050/62, 13385/62 4663/62 752/52*, 19116/51*	1.701 1.700 1.7015 1.702 1.7035	41 40 41.5 42 43.5
<i>Moraxella bovis</i>	8561, 9425, 10900 9426	1.7025 1.703	42.5 43
Serum-liquefying, nonhaemolytic moraxellae	9833, 9985, F1, L1 7911 11748 A947(1)	1.703 1.7015 1.702 1.7025	43 41.5 42 42.5
"19116/51" group of oxidase positive rods	5718, 5873, 8134, 19116/51, A608, A1920 5893, 8292, 9893	1.7035 1.703	43.5 43
<i>Neisseria catarrhalis</i>	Ne11, 12910/62, 13016/62, 13074/62, 13135/62, 13430/62, 2833/63, 2982/63, 7889/63, 1163/64, 1179/64, 2424/64 8176 4103*	1.701 1.702 1.7025	41 42 42.5
<i>Neisseria caviae</i>	10293, 14659	1.7045	44.5
<i>Neisseria ovis</i>	37/59, 917/60 199/55	1.7045 1.705	44.5 45
<i>Neisseria cinerea</i>	165/61, 159/62	1.709	49
<i>Neisseria flavescens</i>	8263 13115 13120	1.7065 1.707 1.7075	46.5 47 47.5
<i>Achromobacter lwoffii</i>	17985 17987 881/57 8858/62	1.703 1.7035 1.7015 1.698	43 43.5 41.5 38
<i>Achromobacter anitratus</i>	8, 9	1.6995	39.5
<i>Achromobacter haemolyans</i>	742/56 2408/57 2181/60 17998	1.705 1.7015 1.6995 1.701	45 41.5 39.5 41

* Deviating in terms of transformation (see Table 1)

TABLE 3

Range of G + C Content of Entities Considered equivalent to Species on the Basis of Streptomycin Resistance Transformation (Ratios of Inter to Intrastrain Transformation 10⁻³ or Higher see Table 1) and Interspecies Streptomycin Resistance Transformation within Arbitrary Groups

Arbitrary group	Assumed entity equivalent to a species	Range of per cent G + C	Range of ratios of inter to intrastrain transformation between entities of the same group
A	1 <i>M nonliquefaciens</i> *	40-42	
	2 <i>M bovis</i>	42.5-43	1.5 10 ⁻³ - 7.5 10 ⁻³
	3 Serum liquefying nonhaemolytic moraxellae	41.5-43	1.2 10 ⁻³ - 1.8 10 ⁻²
	1 <i>M nonliquefaciens</i> *	40-42	1.0 10 ⁻³ - 5.2 10 ⁻³
B	19116/51 group	43-43.5	
C	1 <i>V. caviae</i>	44.5	
	2 <i>V. ovis</i>	44.5-45	4.9 10 ⁻⁴ - 5.6 10 ⁻⁴
D	1 <i>V. catarhalis</i> (typical)	41-42	
	2 <i>V. catarhalis</i> 4103	42.5	5.4 10 ⁻⁴ - 4.6 10 ⁻³
F	1 <i>V. cinerea</i>	49	
	2 <i>V. flavescens</i>	46.5-47.5	5 10 ⁻² 6 10 ⁻² †

M = *Moraxella* *V* = *Veisseria*

* The deviating strain 752/52 excluded Strain 19116/51 also excluded and moved to the 19116/51 group

§ One way transformation reactions

† Semiquantitative estimates expressed in quantitative terms

During the further study of the 19116/51' group (Boure 1965d) it was found that nitrate reduction varied within the genetically homogeneous group and therefore could not be used for distinction from the presumably regularly nitrate reducing *Moraxella nonliquefaciens*. It was shown that other conventional characteristics followed the transformation pattern and inter also DNA base ratios (Table 2).

Some of these examples show that weighting of conventional characteristics in a group of bacteria may be put on a sound basis by the application of quantitative transformation. An easily performed high frequency transformation experiment may also be employed directly in some cases of problematic diagnosis when a system of genetically arranged microbes is available.

A first degree transformation compatibility (ratios of inter to intra strain transformation to streptomycin resistance ranging from 1/3 to 1) may be taken as an indicator of widely distributed homologies of the genomes in question (see Bourre 1964b 1965b d and Tables 1 and 2). Findings by other investigators (see previous discussion) have even shown that if reciprocal streptomycin resistance transformation ratios are 1/10 or higher the strains in question have similar affinities also

by other means, and species identity has been discussed also on the basis of other criteria alone

For several reasons an entity with compatibility ratios from 1/10 to 1 in streptomycin resistance transformation (preferably reciprocal) may be suggested as a base-line above the strain concept in bacterial classification. Delimitation of a species will be based on this genetically defined entity in the following discussion. Within such a species concept variation of G + C content is 2 per cent at maximum (Table 3).

On the basis of the transformation studies as a whole one is forced to look at the reactions between *Neisseria cinerea* and *Neisseria flavescens* and also between the fastidious *Moraxella nonliquefaciens* (Bovre 1964b), *Moraxella bovis* and the serum-liquefying, nonhaemolytic moraxellae as indicating particularly close relationships between species (Table 3). The affinities of the two former are comparable to those found between *Neisseria flavescens* and the saccharolytic neisseriae (Collins & Cunningham 1961), and the compatibilities between the moraxellae may be compared with the reactions between some of the closest related *Streptococcaceae* and between *Haemophilus influenzae* and *Haemophilus parainfluenzae* (see previous section of this paper). Some other interactions deserve special consideration, not least because they partly point to the significance of the compatibilities discussed above. The reactions between ordinary *Neisseria catarrhalis* strains and the nitrate negative *Neisseria catarrhalis* 4103 (Bovre 1965b) are of a similar order or slightly less pronounced than those between moraxellae. *Neisseria caviae* and *Neisseria ovis* are a little more distant from each other than are the moraxellae in the same terms. Nevertheless they are very similar in conventional terms (Bovre 1965b) and their overall base compositions are identical.

The only other species combination which reveals ratios of compatibility higher than the *Neisseria caviae*/*Neisseria ovis* ratios is the combination *Moraxella bovis*/*Neisseria ovis* (Table 4). Important is here the apparent existence of DNA regions with more pronounced homologies than the region bearing the streptomycin locus (Bovre 1967a). It is tempting to compare the reactions between *Moraxella bovis* and *Neisseria ovis* with some interactions of *Streptococcaceae*, for instance (see previous discussion).

Deduction from transformation compatibility becomes progressively more difficult with the gradually decreasing ratios of inter- to intra-strain transformation. The relative magnitude of interactions inferior to $5 \cdot 10^{-4}$ may be ordered as follows (Table 4). The affinities of *Moraxella liquefaciens* and the fastidious *Moraxella nonliquefaciens* to *Neisseria ovis* are partly close to the *Neisseria caviae*/*Neisseria ovis* interactions. The affinities in these terms between *Neisseria catarrhalis* and all three unequivocal *Moraxella* entities, between *Neisseria ovis* and *Neisseria catarrhalis*, between *Neisseria ovis* and the "19116/51" group, between the "19116/51" group and the three unequivocal *Morax-*

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	Entity§ (species)	Strain	Group§	Entity‡ (species)		
C	2	199/55	A	2	6.2	10 ⁻⁴ - 1.1 10 ⁻³
C	2	199/55	A	3		2.4 10 ⁻⁴
C	2	199/55	A	1	7.5	10 ⁻⁵ - 1.0 10 ⁻⁴
D	1	Ne11	A	1-2 3	1.8	10 ⁻⁵ - 7.4 10 ⁻⁵
C	2	199/55	B	1 2	3.0	10 ⁻⁵ - 4.6 10 ⁻⁵
C	2	199/55	B		2.2	10 ⁻⁵ - 4.4 10 ⁻⁵
H		5873	A	1 2 3	0	10 ^{-6†} - 4.0 10 ⁻⁵
D	1	Ne11	H		0	10 ^{-6†} - 4.0 10 ⁻⁵
D	1	Ne11	C	1-2	9.8	10 ^{-6†} - 3.4 10 ⁻⁵
D	1	Ne11	<i>Achromobacter</i>			7 10 ^{-6†*}
C	2	199/55	<i>Achromobacter</i>			2 10 ^{-6†*}
B		5873	<i>Achromobacter</i>			1 10 ^{-6†*}
A			<i>Achromobacter</i>			nt
A			E	2		nt
B			F	2		nt
C			F	1-2		nt
D			F	1-2		nt

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‡ See Table 3 *Achromobacter* added, see Table 2

† Semiquantitative estimates expressed in quantitative terms

* Maximum ratio observed employing all 10 donors

nt = Transformants not observed in semiquantitative experiments of very high sensitivity

ella entities and between *Neisseria catarrhalis* and the "19116 51" group are rather similar and lower, but still clearly observable. The relation to *Neisseria catarrhalis* of *Neisseria caviae* is of the same order as that of *Neisseria ovis*. Typical *Neisseria catarrhalis* has the same relation to *Neisseria ovis* as the deviating strain 4103. *Achromobacter* strains have affinities to *Neisseria catarrhalis* which generally are distinctly lower than the ones mentioned above but which also partly overlap the activity of the "19116 51" group on the same recipient in simultaneous experiments (Borre 1967 b). There is a slight activity of *Achromobacter* donors on the *Neisseria ovis* recipient, which generally is doubtful (Borre 1967 b). The activity of *Achromobacter* on a recipient from the "19116 51" group is also very slight. The latter activity is distinctly lower than that exerted by unequivocal *Moraxella* on the same recipient (but see Borre 1967 b). No activity has been observed on *Moraxella* of *Achromobacter* donors, and no interaction of *Neisseria flavescens* with other oxidase positive recipients under the most sensitive test conditions.

That a motile, *Pseudomonas* like donor was slightly active in transformation of *Neisseria catarrhalis* (Borre 1967 b) is confusing. However, this reaction was distinctly less frequent than any other positive reaction observed. Only the future may show the relevance of

by other means, and species identity has been discussed also on the basis of other criteria alone

For several reasons an entity with compatibility ratios from 1/10 to 1 in streptomycin resistance transformation (preferably reciprocal) may be suggested as a base-line above the strain concept in bacterial classification. Delimitation of a species will be based on this genetically defined entity in the following discussion. Within such a species concept variation of G + C content is 2 per cent at maximum (Table 3).

On the basis of the transformation studies as a whole one is forced to look at the reactions between *Neisseria cinerea* and *Neisseria flavescens* and also between the fastidious *Moraxella nonliquefaciens* (Bovre 1964 b), *Moraxella bovis* and the serum-liquefying, nonhemolytic moraxellae as indicating particularly close relationships between species (Table 3). The affinities of the two former are comparable to those found between *Neisseria flavescens* and the saccharolytic neisseriae (Callin & Cunningham 1961), and the compatibilities between the moraxellae may be compared with the reactions between some of the closest related *Streptococcaceae* and between *Haemophilus influenzae* and *Haemophilus parainfluenzae* (see previous section of this paper). Some other interactions deserve special consideration, not least because they partly point to the significance of the compatibilities discussed above. The reactions between ordinary *Neisseria catarrhalis* strains and the nitrate negative *Neisseria catarrhalis* 4103 (Bovre 1965 b) are of a similar order or slightly less pronounced than those between moraxellae. *Neisseria caviae* and *Neisseria ovis* are a little more distant from each other than are the moraxellae in the same terms. Nevertheless they are very similar in conventional terms (Bovre 1965 b) and their overall base compositions are identical.

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Deduction from transformation compatibility becomes progressively more difficult with the gradually decreasing ratios of inter- to intra-strain transformation. The relative magnitude of interactions inferior to 5-10% may be ordered as follows (Table 4). The affinities of *Moraxella liquefaciens* and the fastidious *Moraxella nonliquefaciens* to *Neisseria ovis* are partly close to the *Neisseria caviae*/*Neisseria ovis* interactions. The affinities in these terms between *Neisseria catarrhalis* and all three unequivocal *Moraxella* entities, between *Neisseria ovis* and *Neisseria catarrhalis*, between *Neisseria ovis* and the "19116 51" group, between the "19116 51" group and the three unequivocal *Morax-*

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Selected experiments generally one way in the most effective transformation direction

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That a motile *Pseudomonas* like donor was slightly active in transformation of *Neisseria calarrhalis* (Bire 1967 b) is confusing. However this relation was distinctly less frequent than any other positive reaction observed. Only the future may show the relevance of

this observation. In any case, the many non reacting donors of *Alcaligenes Pseudomonas*, *Neisseria meningitidis* *Neisseria flavescens* etc (Bovre 1967 a, b) show that there are zero endpoints of the distribution of streptomycin resistance transformation in the vicinity of *Moraxella* even when the most sensitive methods are used.

The species concept proposed above should not be considered as a genospecies but as a genetically defined taxonomic species. If a genus species is taken to indicate a group of organisms between which genetic transfer is possible (Ravin 1960) it now seems that such a group may be very wide.

Transformation Compatibility and DNA Base Composition Applied to Some Actual Taxonomic Questions

Affinities between oxidase positive, immotile rods The fastidious *Moraxella nonliquefaciens* (Bovre 1964 b) *Moraxella bovis* (Bovre 1965 a) and the serum liquefying, nonhemolytic moraxellae (Bovre 1965 c) may be said to represent three particularly closely related species in terms of transformation compatibility and DNA base composition. Thus the interrelations of the organisms described by Petit (1899) by Scarlett (1916) and by Jones & Little (1923) are clearly indicated. If the strains of *Moraxella lacunata* employed (Bovre 1965 c) are representative of the organisms described by Morax (1896) and Axenfeld (1897), the transformation results (Bovre 1965 c) and DNA base data indicate that these organisms and those of Petit are varieties or types of the same entity. This is in accordance with the opinion expressed by Axenfeld (1929). Another variety of the same entity seems to be the organisms isolated by Ryan (1964) from guinea pig conjunctiva (Bovre 1965 c).

An important implication is that the concept *Moraxella nonliquefaciens* (in its common sense) is heterogeneous in corroboration with the results of conventional tests performed by Mannheim & Stenel (1962). Whether the original description of *Bacillus duplex non liquefaciens* (Scarlett 1916) was based on the fastidious ones with the closest relation to other moraxellae (as assumed above) or on strains similar to the 19116/51 group (growing fairly well on Hugh & Lefson's medium and feebly in citrate media see Bovre 1965 d) cannot be decided with certainty. In either case the 19116/51 group is clearly distinct from the fastidious *Moraxella nonliquefaciens*. The results of DNA base determinations show a very uniform deviation between the two and their transformation compatibility is very low. The '19116/51' group should therefore be considered as a separate species, probably with some taxonomic relation to moraxellae. The name *Mima polymorpha* var. *oxidans* has in some instances been used on strains probably belonging to the 19116/51 group but the same name has also been applied to dissimilar organisms (Bovre 1965 d). The name is not recommended for the 19116/51 group (see below).

It is known from preliminary investigations that there are oxidase positive immotile rods which have little or no compatibility with any of the oxidase positive rods mentioned including the "19116a1" group. Thus the urease positive strain 7a2 (Bovre 1964b) and organisms received from the late Miss E. O. King may constitute another separate entity.

Since the descriptions of *Bacillus duplex non liquefaciens* (Scarlett 1916) and *Vima polymorpha* var. *oxidans* (De Bord 1942) both are inadequate and authentic strains are unavailable any attempt to prove their identity will inevitably be fruitless. It is felt that *Vima polymorpha* var. *oxidans* and the fastidious *Moraxella nonliquefaciens* are not identical since genetic studies as yet have provided no evidence of identity of the latter and strains with the former designation and also because *Vima polymorpha* var. *oxidans* originally was described as luxuriantly growing with white colonies. For discussion of the standing in nomenclature of the names *Vimeae*, *Vima* and species epithets *Henriksen* (1963b) and *Pickett & Vanclark* (1966) should be consulted.

Affinities within *Neisseria* The conclusion of *Callin & Cunningham* (1964a) that the nitrate negative strain *Neisseria catarrhalis* 4103 and *Neisseria cinerea* might be identical has not been verified (Bovre 1966b). Although transformation between this strain and other *Neisseria catarrhalis* strains has given similar results in both investigations (in itself a good control of the strains employed and the reproducibility of the method) direct testing of *Neisseria cinerea* strains revealed their distinctiveness from strain 4103 and their probably close relations to *Neisseria flavescens*. DNA base composition data are corroborative. The strain 4103 may represent a species close to *Neisseria catarrhalis*. Any consistent characteristic of this eventual species cannot be suggested with only one strain.

There seems to be a rather close relationship between *Neisseria citris* and *Neisseria catiae*. Their DNA base compositions are identical. These organisms also have some relations to *Neisseria catarrhalis* in terms of transformation and in biochemical and cultural tests (Berger 1962; Bovre 1966b). Some taxonomic distance between the latter and the two former is indicated by their low transformation compatibility and by their deviating DNA base ratios. However, the suggestion that *Neisseria catiae* may be a close relative of the pigmented saccharolytic neisseriae (*LaVauchua & Pellerin* 1966) does not at all fit with the present investigation. It is possible that strains called *Neisseria catiae* represent different entities. In that case it is important to note that the present study is with the type strain of the species *Bovre* (1966b).

The DNA base ratios of *Neisseria citris* and *Neisseria catiae* are identical. Between (*Neisseria catarrhalis*, *Neisseria citris*, *Neisseria catiae* and *Neisseria cinerea*) and (*Neisseria citris*, *Neisseria flavescens*) and (*Neisseria catiae* and *Neisseria flavescens*) the relationship be-

tween the two groups (see *Bovre 1965b*). It may therefore seriously be doubted whether all these organisms should be placed in one genus, as is now the case (*Breed, Murray & Smith 1957*). *Callin & Cunningham (1961)* arrived at a similar conclusion as regards *Neisseria catarrhalis* and its distinction from group I neisseriae (see previous discussion of this report). Thus, results are largely corroborative and supplement each other on this point.

Affinities between *Moraxella* and *Neisseria* The closeness in terms of overall DNA base composition and partly rather pronounced transformation compatibility of moraxellae and not yellow-pigmented, saccharolytic neisseriae (including *Neisseria caniae*), indicate that the two assumed entities have important relations. They should probably be placed in the same family of the present taxonomic system, as suggested by *Henriksen (1952)* on the basis of conventional criteria.

Affinities between oxidase positive and oxidase negative organisms *Henriksen (1952)* proposed that the genus *Moraxella* (*Lwoff 1939*) should not be supplemented with oxidase negative organisms. *Brisou & Morichau-Beauchant (1952)* and *Brisou, Morichau-Beauchant & Gimenez (1953)* shared the opinion that *Moraxella lwoffii* (*Andreou 1940*) does not belong to genus *Moraxella*. *Piechaud, Piechaud & Second (1956)*, however, held the attitude that *Moraxella* includes oxidase negative species. So far, the view of *Henriksen* and *Brisou* and collaborators seems correct.

Close relationships across the boundaries between oxidase positive and oxidase negative organisms seem possible in terms of overall DNA base composition. It is noted that the two *Achromobacter lwoffii* strains with greatest similarity to the '1911651' group have the same G + C content as the latter (see *Bovre 1967b*). The results of transformation experiments, however, do not support this eventuality. There is no indication of a species (*Mima polymorpha*) with an oxidase positive and an oxidase negative variety, as first introduced by *De Bord (1942)* and as mentioned no indication that *Moraxella lwoffii* belongs to the same genus as the fastidious moraxellae. It is not known, however, whether genetic reactivity of the order observed between *Achromobacter* and the "1911651" group, and between the former and *Neisseria catarrhalis*, is indicating some distant relationship. Further studies on this point ought to have high priority.

Comment A complete taxonomic proposal at this stage is considered premature. Many of the above considerations are preferably left as working hypotheses for future investigations in the field.

It may be argued that the proposed species entity is too rigidly delimited and that DNA base similarity and transformation interactions of the order observed between the three unequivocal *Moraxella*

entities suggest species identity (still with the "19116-51" group as a separate species). This attitude would at least partly be in accordance with the opinion of numerous authors. The same theoretical line would lead to the suggestion that *Neisseria flavescens* and *Neisseria cinerea* represent the same species which does not seem unreasonable. The exclusion of strain 4103 from *Neisseria calarrhalis* would be prevented. *Neisseria caniae* and *Neisseria ovis* also would possibly be considered as varieties of the same species. In other parts of the classification system however analogous deductions would lead to more radical changes although still not very unreasonable. For instance *Neisseria meningitidis* would be a variety of a species containing all hitherto examined yellow pigmented and/or saccharolytic neisseriae. Species distinction between pneumococci and some streptococci would be difficult to maintain. In this eventual alternative system the relations between moraxellae and the neisseriae in question would be even more suggestive.

However a conservative attitude is considered the best alternative. One must take advantage of additional information which undoubtedly very soon may be made available.

Differences in conventional terms found between groups suggested on the basis of genetic compatibility and DNA base similarity should be followed up and supplemented for the ultimate construction of a useful key reaction system.

SUMMARY

A review has been given on genetic transformation and overall DNA base composition in taxonomy. Own transformation studies in *Moraxella*, *Neisseria* and some other organisms were summarized and discussed. DNA composition data of the material showed good correlation with the transformation results.

The main conclusions were:

1. *Moraxella nonliquefaciens*, *Moraxella bovis* and the serum liquefying nonhemolytic moraxellae (inclusive *Moraxella lacunata*) may be considered as three particularly closely related species.

2. A distinct group of *Moraxella nonliquefaciens* like organisms provisionally called the "19116-51" group should be considered as a separate species probably with some taxonomic relations to the unequivocal moraxellae. This species is in conventional tests indistinguishable from the fastidious *Moraxella nonliquefaciens* except for its capacity to grow fairly well on Hugh & Leifson's medium and feebly in citrate media. Its G-C content in CsCl buoyant density determinations is of the order 13-13.5 per cent (40-42 per cent for *Moraxella nonliquefaciens* with most strains at 41 per cent). Streptomycin resistance transferation between the "19116-51" group and unequivocal moraxellae has never been observed (0-0.4%) of intra-strain transfer at a frequency whereas *M. nonliquefaciens*

always gives reciprocal ratios above 10^3 with the other two unequivocal moraxellae

3 Absence of genetic compatibility and DNA base similarity between the groups (*Neisseria catarrhalis*, *Neisseria ovis*, *Neisseria caviae*) and (*Neisseria cinerea*, *Neisseria flavescens*) may indicate uncorrect ordering of these organisms in one and the same genus *Neisseria cinerea* and *Neisseria flavescens* should be considered closely related

4 Overlap in terms of DNA base composition and partly also rather pronounced genetic transfer between moraxellae and asaccharolytic not yellow pigmented neisseriae suggest that the two groups have important taxonomic relations and that family distinction between them probably is too rigorous

5 There is at present no evidence of relationship between unequivocal moraxellae and oxidase negative bacteria, inclusive *Achromobacter* (*Moraxella*) *lwoffii*. Weak interactions observed in transformation between *Achromobacter*, the "1911/51" group and *Neisseria catarrhalis* should lead to further investigations, however

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SEROLOGICAL PROPERTIES OF ANTISERA TO *NEISSERIA GONORRHOEAE* ANTIGENS

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In 1950 *Thomas & Vennie* (26) described the indirect haemolysis test, called by them the polysaccharide lysis test, for detection of antibodies in human sera against antigen extracted from gonococci. They proposed to substitute the conventional complement fixation test by the indirect haemolysis test in the diagnosis of gonorrhoea.

Chanarin (3) extracted 2 antigens from smooth strains of gonococci by alkaline hydrolysis of the bacteria. One antigen was detected by its ability to sensitize erythrocytes to the action of gonococcus antiserum. The antigen failed to react in a precipitin reaction and did not fix complement in the presence of antiserum. The second antigen was detected by its ability to fix complement in the presence of antiserum to gonococci. *Chanarin* suggested that the 2 antigens formed a complex antigen on the surface of the gonococcus.

In a previous work the author reported on the gonococcus antigen which sensitizes erythrocytes (19). The antigen was extracted following the procedure of *Chanarin* (3). The indirect haemolysis test was employed for the titration of antibodies against sensitized erythrocytes. The titre of the haemolysis test depended on the antigen dose employed for sensitization, the complement concentration used, and time of incubation. Antibodies against sensitized erythrocytes were found in 55 per cent of blood donor sera. The indirect haemolysis test afforded no advantage over the complement fixation test in the diagnosis of gonorrhoea. A rabbit antiserum and human sera also agglutinated sensitized erythrocytes. The titre of the antiserum in the indirect haemolysis test far exceeded that of the haemagglutination test.

Some properties of antisera obtained by immunization of rabbits with whole gonococci and with sensitized erythrocytes, have been studied. This paper presents the results.

MATERIAL AND METHODS

A *gonorrhoeae* str. Strain 8351/64 isolated from a patient suffering from acute gonorrhoeal u. This strain was selected. The strain was cultured and stored by

Lyophilization Cultivation, harvesting and preparation of the bacterial extract were performed as described previously (19).

Antisera Antisera against strain 8551/64 (designated anti Gc) were prepared according to the immunization schedule described previously (19). Antisera against the antigen which sensitizes erythrocytes (designated anti S\SE (sensitized normal sheep erythrocytes)), were obtained by immunizing 2 rabbits with sensitized erythrocytes. The erythrocytes were sensitized as previously described (19), thoroughly washed and prepared as a 10 per cent suspension. One ml was injected intravenously thrice weekly for four weeks. As the antibody response was relatively weak, a new series of three injections was given after an interval of 4 weeks. The animals were bled 7 days after the last injection. The sera were stored at -20°C . Before use they were inactivated and absorbed with unsensitized sheep erythrocytes.

Indirect haemagglutination and haemolysis tests The procedures described previously were followed except that the haemolysis test was modified when sera previously treated with mercaptoethanol or absorbed with gonococci were titrated. Before complement was added the sheep erythrocytes in each tube were washed once and resuspended in 0.4 ml volumes.

Complement fixation test When whole gonococci were employed as antigen the test was performed as previously described (19). Using extract of gonococci the procedure was similar except that fixation of the complement took place overnight at 4°C before incubation at 37°C .

Precipitation tests Precipitation in capillary tubes (ring test precipitation) was performed according to Haukenes (11). Dilutions of the bacterial extract were layered over undiluted serum. The results were recorded instantaneously and after $\frac{1}{2}$, 1, 2 and 3 hours incubation at room temperature.

The technique used in the agar gel diffusion tests has been described elsewhere (12). The antigen wells were filled either with a suspension of gonococci containing about 100 mg of bacteria (wet weight) per ml or with undiluted extract. The serum wells were filled with undiluted serum. The plates were kept at 37°C for 1 day followed by incubation at 4°C for 1 or 2 days. The precipitation patterns were read daily.

Agglutination of gonococci Agglutination of live gonococci and gonococci treated with heat or formalin was performed using a slide technique. An 18-20 hours culture was harvested in saline. One portion of the gonococci was resuspended in saline to a density of approximately 10^8 organisms per ml. The second portion was boiled in a water bath for 60 minutes, washed once and resuspended in a similar manner. This washing was necessary to obtain maximal titre, probably because antigens liberated to the fluid phase inhibited agglutination. A third portion of gonococci was treated with 3 per cent formalin at room temperature for 60 minutes, thoroughly washed and resuspended in saline. Twofold dilutions of the antisera were prepared in saline from 1:2. One drop of each serum dilution was mixed with 1 drop of the bacterial suspension on a slide. The slide was rocked for 10 minutes and left on the disk for 5 minutes. The agglutination appeared as numerous fine granules. The titre was given as the reciprocal of the highest serum dilution with marked agglutination.

Absorption Samples of undiluted antisera were absorbed either with live gonococci or with organisms treated by heat at 100°C for 60 minutes. For the absorption of 0.1 ml of serum about 200 mg of bacteria (wet weight) were employed and the mixture was incubated at 4°C for 4 hours.

The extract of gonococci was also sorbed with unsensitized erythrocytes (22). Several absorptions with large doses of erythrocytes were needed. Absorption was considered complete when the extract no longer sensitized erythrocytes to the action of an antiserum.

Mixed agglutination The procedure was adapted from that described by Tønder *et al.* (27) working with mixed agglutination with tissue sections. One drop of a suspension of gonococci was placed in the centre of a coverglass. The preparation was air dried, fixed in formalin and washed according to Høess *et al.* (13). The bacteria were sensitized by placing 1 drop of anti Gc dilute 1:1-4 on the preparation which thereafter was kept at room temperature for 60 minutes, washed and air dried. Microculture slides with a single concavity were filled with a 1 per cent suspension of erythrocytes sensitized as usual with the bacterial extract. The coverglass was placed in such a way that the bacterial preparation was submerged in the erythrocyte suspension. The slides were inverted and kept at 4°C overnight. They

were again turned over and the coverglass was tapped gently around the bacterial preparation to accelerate the detachment of passively adsorbed erythrocytes. The preparation was then examined microscopically at a 100 fold magnification.

Treatment with mercaptoethanol. Equal volumes of undiluted antiserum and 0.2 M β -Mercaptoethanol pH 7.4 were mixed, kept at room temperature overnight and tested for activity (10). Proper controls were included.

Gel filtration. A 3 ml sample of anti Ge was absorbed with unsensitized sheep erythrocytes and applied to a Sephadex G 200 gel bed of 3.5×45 cm. The gel filtration was performed according to Flodin & Kallander (9) employing 0.05 M potassium phosphate buffer pH 7.3 containing 0.4 M NaCl and 0.02 per cent sodium azide. The UV absorption of the effluent was recorded at 254 m μ in a Unicord absorptiometer (LKB Produkter Stockholm Sweden). The fractions were tested for activity in the indirect haemagglutination and haemolysis tests.

EXPERIMENTS AND RESULTS

Bacterial Agglutination, Complement Fixation, Indirect Haemagglutination and Haemolysis

In preliminary experiments the titres of the sera in the various tests were determined using varying concentrations of the antigens. In the final tests antigen concentrations were chosen so as to give maximal titres.

TABLE 1
Titres of Anti Ge and Anti S\SE in the Various Tests

Serologic tests	Antigen	Anti Ge	Anti S\SE
Bacterial agglutination	Live gonococci	256	<2
	Boiled gonococci	128	32
Complement fixation	Whole gonococci	256	32
	Extract of gonococci	128	16
Indirect haemagglutination	"	512	64
Indirect haemolysis	"	8192	512

Table 1 shows the results obtained with anti Ge and anti S\SE. Anti Ge reacted in the bacterial agglutination test with both live and boiled gonococci. With boiled gonococci the titre seemed to be slightly lower in accordance with the finding of Danielsson (4, 6). Anti S\SE did not react in the agglutination test with live gonococci but boiled gonococci were agglutinated. The agglutination test with formalinized bacteria allowed no conclusion because the bacteria agglutinated spontaneously to a considerable degree. No spontaneous agglutination occurred with live or boiled gonococci.

Anti S\SE absorbed with unsensitized sheep erythrocytes was moderately anti-complementary, presumably caused by soluble complexes of antigens and antibodies in the absorbed serum. This was circumvented by absorbing the serum with sheep erythrocyte stromata and centrifuging at high speed. Anti Ge and anti S\SE reacted in the complement fixation test no matter whether whole gonococci or the extract was the antigen (Table 1).

Twofold dilutions of unabsorbed extract and of extract absorbed with

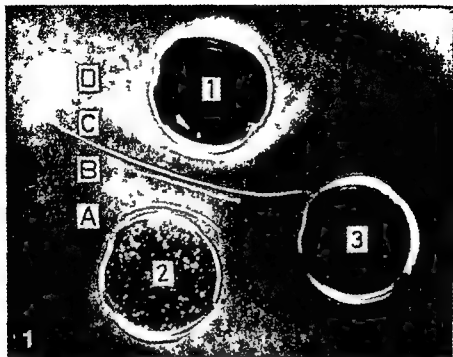


Fig. 1

Precipitation pattern formed by gonococci and antisera. Gonococci: anti Ge and anti SNSC in wells 1, 2 and 3 respectively. Precipitation lines A, B, C and D. See text. Line A and B artificially intensified.

sheep erythrocytes were prepared. The complement fixing ability of the samples in the presence of antiserum was determined using a constant amount of anti Ge. The complement fixing activities of the samples were essentially similar, thus showing that the extract contained one or more antigens in addition to the antigen which sensitizes erythrocytes.

Both antisera reacted in the indirect haemagglutination and haemolysis tests and with considerably higher titres in the latter.

Following absorption of anti SNSC either with live or boiled gonococci, the antiserum no longer agglutinated heat-treated gonococci and the reactivity with sensitized erythrocytes was abolished.

The sera obtained from both rabbits immunized with sensitized erythrocytes behaved similarly in the various tests. Preimmune sera were negative.

Precipitation

Precipitation in capillary tubes with undiluted anti Ge was obtained with dilutions of both unabsorbed extract and extract absorbed with erythrocytes up to 1:128. When tested against undiluted anti SNSC, precipitation appeared up to dilution 1:4 of the extract only.

Suspension of gonococci and the extract were examined by double

diffusion in agar against anti Gc and anti S\SE Fig 1 shows the principal precipitation lines appearing with gonococci in the antigen well Undiluted anti Gc formed 4 precipitation lines referred to as A B C and D Line A formed near the serum well and tended to disappear into the serum basin Midway between the antiserum and antigen wells 2 relatively straight precipitation lines appeared (line B and C) The fourth precipitation line line D formed near the antigen well This line fused with the only precipitation line formed by anti S\SE

With extract in the antigen well anti Gc formed line A only No precipitation line was formed with anti S\SE and the extract

Mixed Agglutination

When smears of gonococci are incubated with antiserum antibodies react with antigens on the surface of the bacterial cell After removal of the serum fixed antibodies are detected with an indicator system which in this case was erythrocytes sensitized with the extract Antibodies bound to the bacteria also react with sensitized erythrocytes and thus function as a linkage between the erythrocytes and the bacterial smear The indicator system employed detects only the antigen which sensitizes erythrocytes

Fig 2 shows the picture of the microscopical readings at 140 fold and 560 fold magnifications The bacterial preparation is covered with erythrocytes while only few erythrocytes attach to the coverglass (Fig 2a) In accordance with the grading used by *Tonder et al* (21) Fig 2a shows a moderately positive reaction (+2) Fig 2b visualizes the arrangement of single erythrocytes at the periphery of the bacterial smear Erythrocytes adhere closely to aggregates of bacteria

In control preparations with sensitized erythrocytes and pre immune serum or unsensitized erythrocytes and antiserum no erythrocytes attached to the bacterial smears

Reduction by Mercaptoethanol, Gel Filtration

The haemagglutinating activity of anti Gc and anti S\SI was only slightly influenced by treatment with mercaptoethanol

On the other hand the haemolysis titres were decreased to about the same as the haemagglutination titres The results indicated that antibodies against sensitized erythrocytes belong to both γM and γG globulins (8, 10)

Fig 3 shows the results obtained by gel filtration of anti Gc The first peak of the elution curve corresponds to fractions containing mainly γM globulin the second one mainly γG globulin (11) This was verified by treatment of the fractions with mercaptoethanol

The activity of the fractions was tested with haemagglutination and haemolysis tests With most γG globulin fractions the titre of the haemagglutination was higher than that of the haemolysis

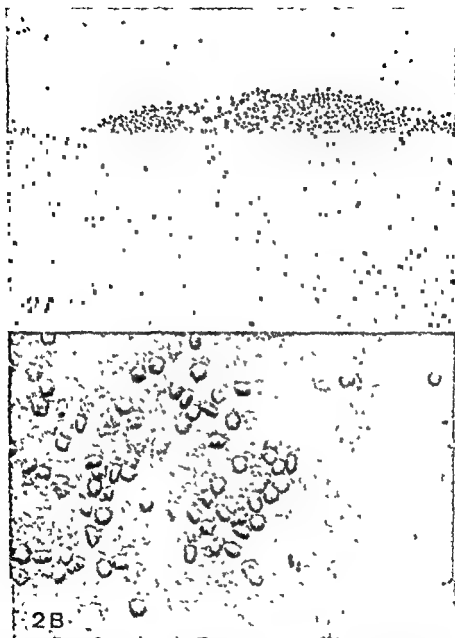


Fig 2

Mixed agglutination with a smear of gonococci on a coverglass

- a 140 fold magnification The smear of gonococci is covered with erythrocytes, while only a few erythrocytes attach to the coverglass
- b Appearance at 560 fold magnification at the periphery of the preparation Sensitized erythrocytes crowded around aggregates of bacteria

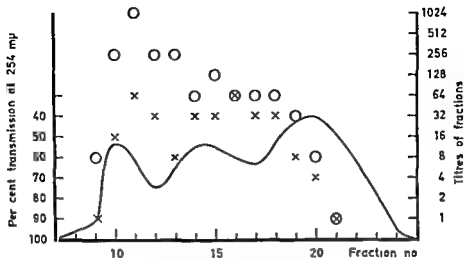


Fig 3

Serologic activity of fractions obtained by gel filtration of rabbit anti Gc serum on Sephadex G 200 column

- Per cent transmission at 254 mμ
 x Titres in indirect haemagglutination test
 o Titres in indirect haemolysis test

molysis test was double the haemagglutination titre. Most γ M globulin fractions reacted with 16 times higher titres in the haemolysis test than with titre difference of the same order as with unfractionated anti serum.

TABLE 2

Titres in Haemagglutination and Haemolysis Tests of Mixtures Containing Varying Quantities of a γ M and a γ G Globulin Fraction of Antiserum

Mixtures titrated		Indirect Haemagglutination	Indirect Haemolysis
γ M fraction	γ G fraction		
10	10	32	1024
8	8	32	128
6	6	64	256
4	4	64	256
2	2	64	512
0	0	64	1024

The influence of antibodies belonging to γ M globulin and γ G globulin on the indirect haemagglutination and haemolysis tests was investigated by varying the concentration of the globulins. Samples from 1 γ M and 1 γ G globulin fraction were mixed as shown in Table 2 and the reactivity of the mixtures against sensitized sheep erythrocytes was determined. The haemagglutinating capacity of the 2 fractions was only

slightly different, and hence the titres of the mixtures in the haemagglutination test increased from 32 to 64 only with increasing volumes of the γ M-fraction in the mixtures. On the other hand, the titres in the haemolysis test increased from 64 to 1024 as the volumes of the γ M-fraction increased. The results indicate a different behaviour of γ M- and γ G-globulin antibodies in the indirect haemolysis test.

DISCUSSION

Rabbits immunized with sensitized erythrocytes formed antibodies against the adsorbed antigen. However, the antibody response was relatively weak as compared to that obtained when live gonococci were injected.

Anti-Gc reacted in the bacterial agglutination test with live and heat-treated gonococci (boiled for 60 minutes). Anti-SNSE did not agglutinate live gonococci, but boiled gonococci were readily agglutinated. The antigen reacting with anti-SNSE is thermostable. Anti-SNSE thus resembles antisera against somatic O antigens of certain enteric bacilli, which do not agglutinate unless the organisms are treated with heat (16). Boor & Miller (1) immunized rabbits with glycolipid from gonococci and meningococci and obtained antisera which exhibited excellent precipitating but mediocre agglutinating power against homologous strains. Deacon (7) observed inagglutinability of freshly isolated strains of gonococci in antisera prepared against heat-treated cultures. He suggested that K-like antigens (labile surface antigens) act as a protective cover over underlying, thermostable, somatic antigens, and prevent agglutination. The K antigen was readily lost in subcultures.

The gonococcus strain employed in this study had been subjected to several subcultures and hence a K-antigen was probably no hindrance for agglutination with anti-SNSE. The reactivity of anti-SNSE against sensitized erythrocytes disappeared after absorption of the antiserum with live gonococci. The antibodies in anti-SNSE thus reacted with live gonococci but treatment of the bacteria with heat was necessary to render them agglutinable by anti-SNSE.

Chanarin (3) did not demonstrate complement-fixing activity of the erythrocyte sensitizing antigen in the presence of antiserum. However, complement was fixed in the presence of anti-SNSE regardless of whether the extract or whole gonococci was the antigen. Unchanged complement-fixing activity in the presence of anti-Gc of extract absorbed with erythrocytes, established the existence of at least one additional antigen in the extract. This finding agrees with the observations of Chanarin (3).

Precipitation in capillary tubes between anti-Gc and unabsorbed and absorbed extract, furnished further evidence for the existence of additional antigen(s) in the extract. This antigen(s) failed to adsorb to erythrocytes. In gel diffusion tests, the additional antigen formed a

precipitation line line A near the serum well with anti Gc Chanarin (3) did not demonstrate precipitation between the extract of gonococci and various antisera

Only weak precipitation occurred between anti S\SE and the extract in capillary tubes and no precipitation line was formed in gel diffusion tests Thus the sensitizing antigen in the extract seemed to be a weak precipitinogen only The precipitating property of anti S\SE was clearly demonstrated in gel diffusion tests with whole gonococci A precipitation line line D formed near the antigen well Danielsson (6) divided the precipitation lines formed by gonococci treated with ultrasonic and antisera against formalin killed gonococci into 4 groups referred to as A B C and D The precipitation line formed by anti S\SE and whole gonococci probably corresponds to group D

Two findings indicate a superficial location in the bacterial cell of the erythrocyte sensitizing antigen 1 The results obtained in the mixed agglutination test The mixed agglutination procedure can detect only antigens localized on the cell surface not those present inside the cell (27) 2 Disappearance of reactivity against sensitized erythrocytes of antisera absorbed with live gonococci

Mixed agglutination technique has mainly been employed in studies on tissue antigens and has previously not been reported with bacterial preparations on slides The principle of mixed agglutination resembles that of immunofluorescent staining which has come into use as a method for demonstrating gonococci in smears from patients (5 7, 23) A similar application of the mixed agglutination technique probably remains a topic for investigation

In the indirect haemagglutination and haemolysis tests anti Gc reacted with considerably higher titres than anti S\SF (Table 1) The titres of the sera in the haemolysis test exceeded the haemagglutination titres 16 and 8 times respectively Essentially similar differences between haemagglutination and haemolysis titres were found with sheep erythrocytes which had adsorbed antigen from *Escherichia coli* and *Salmonella* and homologous rabbit antisera (20 21)

These findings indicated that antibodies against sensitized erythrocytes belong to both γM and γG globulins 1 Reduction of antiserum by mercaptoethanol affected the reactivity of the serum to a considerable degree (8 10) 2 After gel filtration of antiserum activity appeared in fractions known to contain γM and γG globulins respectively (9 17) Antibodies against bacterial antigens of γM class have been found in antisera from several animal species including rabbits (14 15 18)

With most γM fractions the titre of the haemolysis test was 16 fold the haemagglutination titre as compared to a twofold difference with most γG fractions In titration of γM class antibodies the sensitivity of the haemolysis test is thus quite superior to that of the haemagglutination test while the sensitivity of the haemolysis test is only moderately superior in titration of γG class antibodies The results agree with the

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findings of greater haemolytic capacity of γ M- than of γ G-class antibodies in rabbit antisera against sheep erythrocytes (24, 25, 28)

Weinrach *et al* (28) attributed the difference in haemolytic capacity to a different mechanism of complement fixation by antibodies belonging to γ M- and γ G-globulins. Recently *Borsos & Rapp* (2) concluded that a single molecule of γ M globulin at the cell surface is sufficient to bind 1 molecule of C'1a while at least 2 γ G-globulin molecules are needed.

The difference between the titre of the haemagglutination and haemolysis tests depends on the relative amounts of γ M- to γ G class antibodies. Accordingly, one would expect the titre difference to vary with different antisera and with sera obtained at various intervals through the immunization period.

Investigations currently in progress, indicate that anti SNSE contains antibodies of at least 2 different specificities.

SUMMARY

Serologic properties of antisera to whole gonococci (anti-Gc) and antisera obtained by injections of rabbits with erythrocytes which had adsorbed *Neisseria gonorrhoeae* antigen (anti-SNSE), were investigated.

Anti Gc agglutinated live and boiled gonococci. Anti-SNSE agglutinated boiled gonococci only. Anti-SNSE and its homologous antigen exhibited complement-fixing activity. In gel diffusion tests, anti-Gc and whole gonococci formed at least 4 precipitation lines, while anti-SNSE formed a single precipitation line near the antigen well.

Loss of reactivity against sensitized erythrocytes of antiserum adsorbed with live gonococci and the results obtained in mixed agglutination tests, indicated a superficial location in the bacterial cell of the erythrocyte sensitizing antigen.

Antibodies against sensitized erythrocytes belong to γ M- and γ G globulins. In the indirect haemolysis test the antisera reacted with far higher titres than in indirect haemagglutination. This titre difference was attributed to superior sensitivity of the indirect haemolysis test in titration of γ M-class antibodies.

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BRIEF REPORTS

STUDIES ON THE TOXIC EFFECT OF TOXOTOXIN
AND TOXOPLASMA LYSATES

By Filiz K. Pettersen

In 1950 Weinman & Klatchko reported that a cell free peritoneal exudate from mice previously infected with *Toxoplasma gondii* RH strain was toxic to mice when injected intravenously. This toxic effect which was ascribed to toxotoxin was enhanced by incubating the exudate at 56° C for 30 minutes (Woodworth & Weinman 1960) and destroyed by the action of trypsin.

In 1965 Fulton working with cotton rats reported that the effect of toxotoxin was also destroyed by the action of hyaluronidase suggesting that the lethal effect was due to a protein hyaluronic acid complex.

The obvious contamination of *Toxoplasma* antigen preparations with mouse proteins prompted the following investigation the aim of which was to reveal possible toxicity of the contaminants produced by the mouse.

The exudate from 20 g white mice infected with *Toxoplasma gondii* RH strain 5 days previously was used. The parasites were removed by centrifugation at 500 G for 45 minutes. The cloudy supernatant was recentrifuged at 37000 G for 10 minutes giving a clear supernatant liquid which contained precipitating antigens of both *Toxoplasma* and mouse origins. The liquid was incubated at 56° C for 30 minutes and left at 4° C 48 hours later a considerable clot had formed which was obtained as the sediment by centrifugation at 37000 G for 10 minutes. This was dissolved in demineralized water and filtered on a Jenaer G5 sintered glass filter (Ogston 1970). The residue was redissolved in demineralized water twice and filtered giving finally at white residue which was dissolved in 0.9 per cent sodium chloride solution and centrifuged at 37000 G for 10 minutes. The supernatant had a lethal effect on mice which corresponded to that of the original cell free exudate. Unless otherwise stated all the steps in the above procedure were performed at 4° C.

The toxic solution thus obtained was tested in the agar gel diffusion technique (Wadsworth 1962) in which it formed several precipitin lines against rabbit anti mouse serum whereas no precipitin lines were detected against a human anti *Toxoplasma* immunoglobulin preparation.

Incubation with rabbit anti mouse serum at 37° C removed the toxic material from this solution whilst incubation with the human anti *Toxoplasma* immunoglobulin preparation merely diluted the toxic solution.

Thus the toxic material appeared to be of mouse origin rather than a product of the *Toxoplasma* parasite.

In 1964 Lunde & Jacobs described the toxic effect of *Toxoplasma* lysates on rabbits. The parasites were obtained from mouse peritoneal exudate and the toxic effect ascribed to a protein.

In this laboratory the parasites obtained as above were suspended in 0.9 per cent sodium chloride solution and filtered with suction through a Jenaer G1 sintered glass filter. Centrifugation of the filtrate at 500 G for 20 minutes gave a sediment containing the parasites. The sediment was suspended in demineralized water and left at 4° C until the next day when the suspension was centrifuged at 37000 G for 10 minutes giving a clear red liquid. The red lysate. Rabbits injected intravenously with this lysate died within 18 hours. The minimum lethal dose was

found by the Folin phenol reagent (Lowry 1951) to be 60-75 µg protein per kg body weight, which agreed with the findings of Lunde & Jacobs.

The toxicity of these lysates diminished rapidly on standing at temperatures above the freezing point. Storage at -18° C caused some precipitation in the lysates and a solution stored thus for a week had lost more than half of its toxic effect. When however, the lysate solutions were made isotonic before storage at -18° C, no precipitate was formed and the toxic effect was stable over a period of 3 months.

by
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Toxoplasma origins

The eluate was incubated with 20 per cent by volume of a human anti-*Toxoplasma* immunoglobulin preparation for 3 hours at 4° C and centrifuged. The supernatant was toxic to rabbits as before. However, when the same volume of the eluate was

and 3 rabbits previously immunized with whole mouse blood. The results are given in the table.

TABLE
Intravenous Injection of Toxoplasma Lysate in Rabbits

	iv dose ml/kg	time lapse in hours before death
Normal rabbits	0.3	< 18
	0.3	< 18
	0.3	< 18
	0.3	< 18
Immunized rabbits	0.6	> 74
	0.6	24
	0.4	> 26
	0.4	< 18
	0.3	survived

The immunization of the rabbits which was started 8 months previous to this experiment consisted of 6 × one ml intramuscular doses of mouse blood glucose citrate solution and Freund's complete adjuvant (one third of each by volume) injected with about 4 weeks intervals. Apparently this immunization the degree of which was not tested afforded some protection against the toxic effect of the *Toxoplasma* lysate.

In view of these results and those obtained by Lunde & Jacobs this toxic material might prove to be of mouse origin as well as a product of the *Toxoplasma* parasite.

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ARTERIOSCLEROSIS INDUCED BY HYPOXIA

By I Lorenzen and P Helin

In the theories on the aetiology and pathogenesis of arteriosclerosis the effects of anoxia and hypoxia on the arterial wall have been suggested as factors of importance (Hueper 1944). We have been able to demonstrate the development of severe arteriosclerosis in the rabbit aorta following exposure of the animals to repeated periods of hypoxia through three weeks. Male albino rabbits, 5 months of age, weighing about 3 kg were placed in a plastic box, one at a time. The box was covered with plastic foil and air was sucked off through a valve in the bottom. Via another valve in the side of the box pure nitrogen was then inflated until the animal became unconscious and had convulsions. This happened after an average of 50 seconds whereupon the inflation of nitrogen was stopped and atmospheric air inflated instead. About 30 seconds later the rabbit regained consciousness and was able to move. Now and then however artificial respiration had to be given. Immediately after, nitrogen was inflated again thus repeating the procedure. In this way, each animal was exposed to two hypoxic periods in one séance. The entire procedure was performed twice daily.

The study involved 10 experimental animals and 10 controls. The controls were subjected to exactly the same procedure as the experimental animals with the exception that atmospheric air was inflated instead of nitrogen. Three weeks after start the rabbits were killed by intravenous injections of 300 mg of phenobarbital.

Results

In 7 out of 10 experimental animals pronounced gross alterations were observed. The changes consisted of round or elongated hard white plaques mainly localized to the thoracic aorta. In two of the animals the entire thoracic part of the aorta was involved and converted into a stiff tortuous tube. Also aneurysms were observed. In two controls a few small round plaques were observed in the ascending part of the aorta immediately above the aortic valve. These changes were similar to the spontaneous alterations observed in previous studies (Lorenzen 1963). The aortae of the other controls were perfectly normal. Changes similar to those observed in the experimental animals used in the present investigation were not observed in any of the 300 controls used in earlier studies. Microscopic studies of the aortae of the experimental animals showed scattered necrotic calcified lesions in the inner third of the media. These lesions were surrounded by accumulations of amorphous intercellular substance staining metachromatically with toluidine blue indicating acid mucopolysaccharide. The accumulations of metachromatic substance were also observed between the elastic membranes outside the areas of the necrotic lesions. Furthermore collagen deposits were noted partly around the calcified lesions and partly more diffusely scattered in the ground substance (van Gieson-Hansen connective tissue stain). A cellular reaction involving fibroblasts and lymphocytes was observed around the necroses. The elastic membranes of the media showed stretching splitting and fragmentation. In a few animals a slight cellular thickening of the intima was seen usually in the area of medial changes. Two of the control animals showed fibrotic calcified nodules in the subintimal part of the media corresponding to the gross changes mentioned above and to the spontaneous type of lesions described previously. The arteriosclerotic changes induced by hypoxia are of precisely the same type as the changes previously induced in rabbits by injections of adrenalin and L-thyroxine (Lorenzen 1966). The changes are related to the

'Monckeberg-type' of human arteriosclerosis. They may be induced by hypoxia *per se*, or they may be the results of factors which are secondary effects of hypoxia, for example a stimulated release of catecholamines.

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Acta path et microbiol scandinav 69, 159-160, 1967

A REEVALUATION OF THE CHRONIC BIOLOGICAL FALSE POSITIVE PHENOMENON WITH THE FLUORESCENT TREPONEMAL ANTIBODY ABSORPTION TEST

By A. Lassus, K. K. Mustakallio & Aho and T. Puthanen

A negative result of the treponema pall immobilization (TPI) test has been relied upon as the most important serological tests for patients with clinically reactive in the fluores which the serum is first absorbed with Reiter treponemes to remove the group specific treponemal antibodies (1). This prompted us to retest sera from a series of patients classified as chronic BFP-reactors in the previous reports from our clinic (3, 4, 5).

Sera from 57 such patients were available for study. Nine of them had definite systemic lupus erythematosus (2). The FTA ABS test was performed according to the provisional technique published in 1965 by the Venereal Disease Research Laboratory of the Communicable Disease Center, Atlanta.

Fifty of the 57 sera nonreactive to TPI were also negative in the FTA-ABS test, including the sera from the SLF patients. One serum was reactive (2+) and six weakly reactive (1) is presented in the the 50 cases nonre whereas among the 41. The patients, examined for 25 cr neactive tissue dise criteria, one of the 7 patients had definite rheumatoid arthritis

Received 1 xii 66 from the Department of Dermatology University Central Hospital Snellmaninkatu 14 Helsinki 17 Finland and from the State Serum Institute Helsinki Finland. Supported by a grant from the Sigrid Jusélius Foundation Helsinki Finland.

TABLE

Treponema Pallidum Immobilization Negative Cases with Reactivity to the Fluorescent Treponemal Antibody Absorption

Case	Sex	Age	Admission diagnosis	History	I FA ABS
1	♂	59	allergic eczema	drug eruption	reactive
2	♂	44	allergic eczema	—	weakly reactive
3	♀	76	positive STS	thrombophlebitis	weakly reactive
4	♂	65	seborrheic eczema	pleurisy	weakly reactive
5	♀	62	positive STS	rheumatoid arthritis	weakly reactive
6	♀	54	infectious eczema	photosensitivity	weakly reactive
7	♂	58	lichen ruber	myalgia thrombophl	weakly reactive

It is impossible to prove that the TPI negative cases of the present series who showed a weakly reactive IFA ABS test did have latent syphilis. However, the available evidence of these patients as compared with the whole series does not contradict this possibility and we probably are safe in concluding that these patients should not be regarded as definite BIP reactors.

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EPIPHYSEAL GROWTH ZONES IN ACUTE LATHYRISM

Histochemical Study Electrophoresis of Cartilage and Bone

By

E. BERNTSEN

Received 2 June 66

The growth apparatus of tubular bones represent a well-defined anatomical and functional region, and, like other organ systems of the body, it is susceptible to the influence of a number of drugs interfering with the growth process

Since epiphyseal cartilage and spongy metaphyseal bone plates are integrating parts of the growth zone, both of these components were included in this study

Among compounds affecting the growth zones are lathyrogenic substances (Selye 1957) The present paper describes a lathyrotic model, qualitative changes in growth zones and electrophoresis of cartilage and bone

MATERIAL AND METHODS

Throughout experimental studies on lathyrism albino rabbits in their third week of life weighing 200-250 grams were employed Animals and respective mothers were caged batchwise with free access to food and water Control and experimental animals were grouped by weight irrespective of sex at the start of the experiment, so that average body weights of the two groups were as close as possible

A 6 per cent sterile solution of aminoacetonitrile (AAN) Fluka AG Switzerland was made up and adjusted to pH 7.3-7.4 with NaOH A dosage of 15 mg per 100 g body weight per day through 4 days had been found suitable in preliminary experiments Injections were given subcutaneously into the back of the neck, while the controls received a corresponding volume of physiological saline

Carrier free radi sulphate in aqueous solution (The Radiochemical Centre Amersham), was diluted with 0.01 per cent Na₂SO₄ to a final concentration of 1 mCi per ml

Animals were sacrificed on the day after the last injection by an intraperitoneal overdose of Nembutal® From bones of the four extremities epiphyseal cartilages of proximal humerus distal radius and ulna, distal femur and proximal tibia were dissected out under a dissecting microscope By circumcising the periosteum along the epiphyseal line the epiphysis was separated from metaphysis Cartilage was gently scraped off the metaphyseal surface and epiphyseal "cap" After splitting the bone shafts longitudinally marrow was removed and metaphyseal surface cleared of possible cartilage remnants The radicalism of this procedure was controlled by microscopy The metaphyseal bone was then curetted out down to the level of the cortex On metaphyseal plates from corresponding epiphyseal cartilages

Aided by grant from the Danish Rheumatism Association and the Danish State Research Foundation

were removed. Samples were weighed immediately after dissection and dried to constant weight over P_2O_5 in an Edwards tissue drier at a pressure of less than 0.5 mm Hg. After determination of dry weight, cartilage was crushed to a powder of fairly uniform particle size in a small glass grinder. Bone was defatted for one hour in ethanol and for one hour in ether on a mechanical shaker, dried again to constant weight and powdered in a Wiley Mill (micromodel) using a 60 mesh sieve. Decalcification was not performed.

Samples for histological examination were taken from the distal part of the ulna, fixed for 24 hours in a mixture of 8 per cent cetylpyridinium chloride and 10 per cent neutral formalin (Conklin 1963), paraffin embedded, sectioned and stained with Toluidine blue 0.05 per cent at pH 5 for 3 min. and with silver nitrate according to a Kossas method counterstaining with haematoxylin.

The electrophoretic procedure described by Varejmann & Brunish (1965) was followed. Five mg of cartilage powder or 25 mg of bone powder suspended in a Vortex mixer with 200 μ l or 100 μ l of 0.5 N NaOH, respectively. After 24 hours at 4°C samples were centrifuged and 1 μ l of cartilage supernatant or 2 μ l of bone supernatant were applied to cellulose acetate strips. Besides Alcian blue stain for mucopolysaccharides, proteins were stained with 0.0025 per cent nigrosin (w/v) in 2 per cent acetic acid. In some animals approximately 1 μ Ci of ^{35}S - SO_4 per gram body weight had been injected intraperitoneally on the day of the third AAN or saline injection. Autoradiograms were prepared by exposing strips on a Kodak® x ray film for 3–4 weeks.

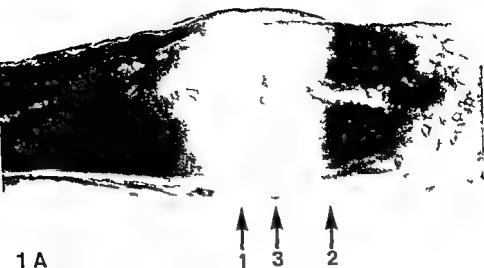
RESULTS

A typical lathyrilic syndrome (Selye 1957) was produced. The first symptom to appear was the ruffled fur, followed by drooping of the ears and a waddling gait with abducted upper extremities. When at rest, animals sat on the rump rather than being supported by the lower extremities. While controls gained weight progressively, the weight increase of experimental animals was arrested.

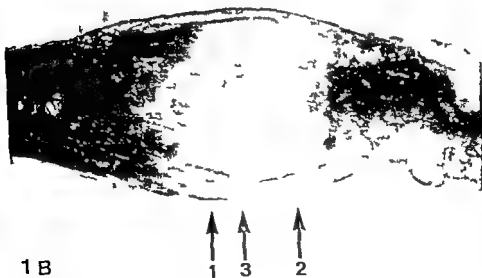
At autopsy, the skin appeared thin, fragile and cut more easily. During removal of bones, a marked muscular atrophy was noted together with diffuse intramuscular bleedings. Proximal humeral and distal femoral epiphyses were invariably the most severely affected (Ponselt & Shepard 1954), occasional slipping always taking place at these sites.

During dissection, control epiphyseal cartilage presented itself as a firm greyish material (Fig 1a, b) resting upon a regular metaphyseal bone plate (Fig 3). Cartilage of experimental animals (Fig 2a, b) appeared loose and swollen, having an oedematous appearance with a definite haemorrhagic tinge. It had a jelly-like consistence, in severe cases amounting to haemorrhagic liquefaction.

The curved metaphyseal plateaus were moulded into more flattened surfaces, and metaphyseal height was reduced to about one third, presenting a disorderly bone structure (Fig 4). A severe osteoporosis (Fig 2b) was noted in the shaft near the growth zone. Lathyrilic bones being definitely more fragile. Periosteum was thickened, oedematous and easily detachable (Fig 2b). No fixed kyphoscoliosis was observed, neither did bowing of extremities nor exostoses develop. The thoraco-abdominal content, in particular the aorta, appeared normal. Costochondral joints were swollen.



1 A



1 B

- Fig 1
a Control rabbit H tal end u d apl p at ng lef C h t n
= crell li periosteum 1 = ag m a lo de " = an n m
cartilage plate to se on la a n erm na la e of a
situated between 2 and 3 6
b Same as Fig 1a but pe h d l all h uch a ap l
in i n Arrow number a 1



2 A

1

3

2



2 B

1

2



Fig 3

Control rabbit Median section through the upper end of the tibia Bone marrow and the entire epiphysis including the cartilage plate has been removed Uniform structure of the spongiosa ($\times 6$)

Fig 2

- i Lathyrus rabbit Distal end of tibia diaphysis is on left The entire growth zone is swollen Periosteum is intact Arrow number 1 as in Fig 1 a ($\times 6$)
- ii Same as Fig 2 a but with periosteum pushed distally through a V shaped incision I — cartilage metaphysis border II — transition from cartilage to germinal cell layer of the cartilage plate I use of the term I epiphyseal slipping periosteum could not be pushed distally a secondary ossification centre Some cartilaginous material is adhering to periosteum Osteoporosis swelling of epiphyseal cartilage and oedema of periosteum is visualized ($\times 6$)



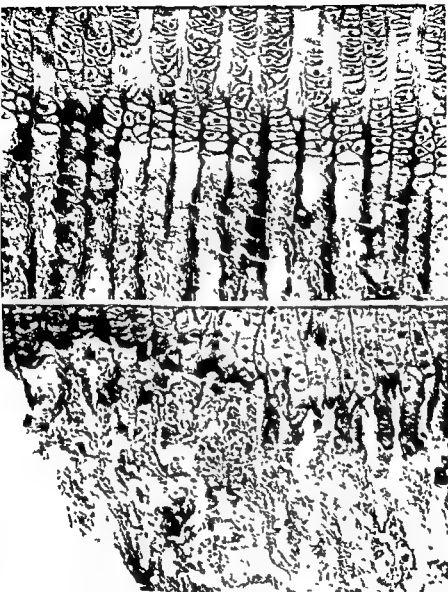
Fig 4

Lithvitic rabbit. Medial section through the upper end of the tibia. Marrow and epiphysis removed as in Fig 3. Metaphysis height is greatly reduced and spongy structure is irregular ($\times 6$).

In one animal regression was followed up to the fifth day and in another animal up to the ninth day after withdrawal of AAN. After a few days body weights started to increase quite rapidly, the fur regained its normal lustre but drooping ears and abducted upper extremities persisted in both animals.

HISTOLOGY

Compared to control cartilage (Fig 5) the lithvitic cartilage plate (Fig 6) appeared swollen with periosteum bulging outwards. An increased proliferation of cells of the maturing zone was observed. Cell clusters were noted at the periphery where ground substance was also more abundant than in the central parts of the plate. A proper hypertrophic zone could not be recognized. Extracellular substance appeared



Figs 7 and 8

- ii Cartilage metaphysis border showing regular formation of toluidine blue stain ($\times 12$)
 i Cartilage metaphysis border showing complete disorganized formation Toluidine blue stain ($\times 12$)

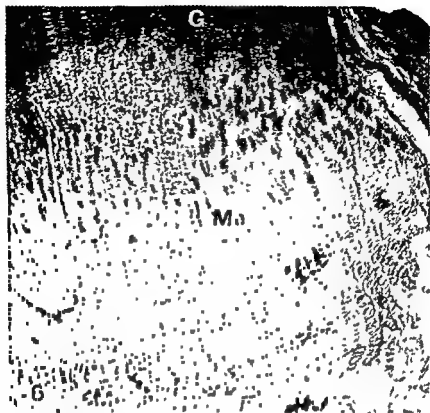


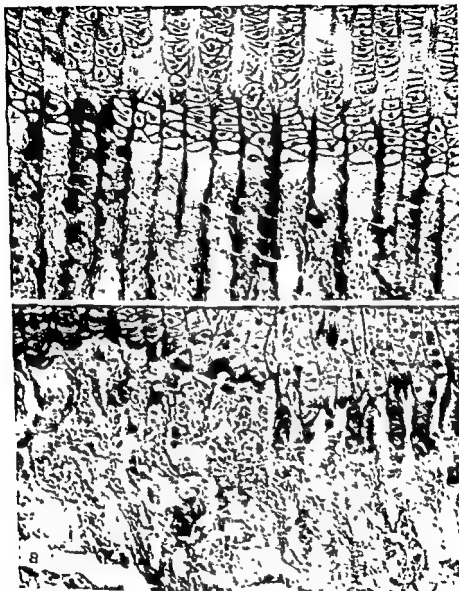
Fig 6

Lathyritic rabbit Zones as indicated in Fig. 5. Maturing zone is widened, there is no definite hypertrophic zone the cartilage metaphysis border is irregular and the metaphysis disorganized Toluidine blue stain ($\times 32$)

When following the evolution of histological alterations from day to day, it was evident that after first AAN-dose, changes had occurred at the cartilage-metaphysis border. The usual uniform pattern of formation of primary spicules was disturbed, leaving the junction with irregular and defectively formed trabeculae (Fig 11). Five days after withdrawal of AAN, this junction was still in a state of complete derangement (Fig 12), but after nine days without the compound an almost normal borderline was seen, with slender, regular and uniform trabeculae (Fig 13).

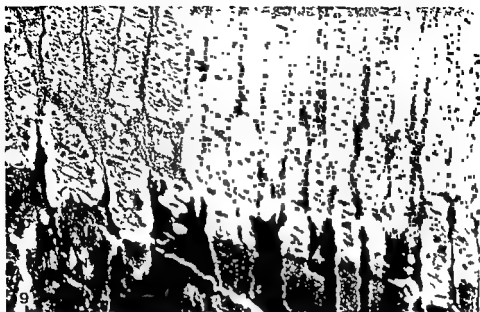
FLUORESCENCE ELECTROPHORESIS

Both in control and experimental cartilages (Fig 14), a distinct chondroitin sulphate (ChS)-band and a distinct though much fainter hyaluronic acid (HA)-band were detected, fractions from controls and experimental animals appearing equally strong. Besides two faint and inconstant bands were observed, one situated between ChS- and HA-



Figs 7 and 8

Fig. 7 Control rabbit. Cast from paraffin-embedded testis showing regular formation of seminiferous tubules. Fig. 8 Cast from paraffin-embedded testis showing complete disorganization ($\times 125$).



Figs 9 and 10

Fig 9 Control rabbit Calcium is concentrating around hypertrophic cells towards the cartilage metaphysis border Van Kossa stain ($\times 125$)

Fig 10 Lathyritic rabbit Compared to Fig 9, a uniform calcification pattern cannot be recognized Van Kossa stain ($\times 125$)

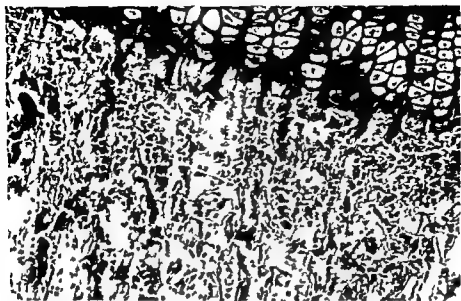


Fig 11

Cartilage metaphysis border of a lathyrotic rabbit 24 hours after a single dose of AAN. Irregular formation of spicules. Toluidine blue stain (X 170)

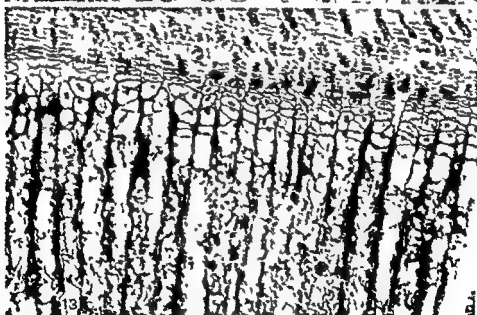
bands the other between HA and the starting line. In metaphyseal bone, the same two major bands were identified (Fig 15). Control and experimental ChS bands again appeared equally strong whereas the HA band of lathyrotic samples was definitely stronger than that of control samples.

Both in cartilage and bone samples ChS-bands were of a slightly slower mobility than standards. Some material was retained at the starting line, especially in case of the bone samples which were also the more viscous. No protein spots were seen except at the starting line. Autoradiograms showed radioactivity of cartilage as well as bone samples to be located in the ChS bands exclusively (Fig 16-17).

DISCUSSION

In establishing the lathyrotic syndrome it was intended to keep the dose as low and the injection period as short as possible. Since variations in food intake must be expected in lathyrism subcutaneous administration rather than ingestion was preferred (Selge 1957). By keeping the duration of the experiment short interference with repair processes was restricted (Karni et al 1961) and the risk of nutritional deficiency minimized (Kuhlmann 1961).

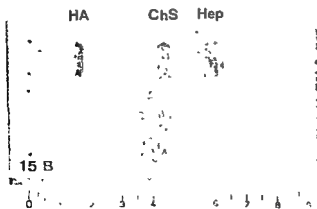
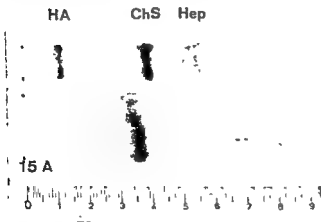
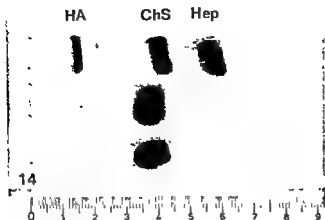
By the dissection technique described it was assured that not only but epiphyseal cartilage and metaphyseal spongiosa was analysed. Therefore all layers of the epiphyseal cartilage were represented.



Figs 12 and 13

Fig 12 (cartilage metaphysis border of a lathyritic rabbit given AAN through 4 days and then sacrificed 5 days after withdrawal of the compound. Formation of spicules is still completely disorganized. Toluidine blue stain ($\times 125$).

Fig 13 (cartilage metaphysis border of a lathyritic rabbit given AAN through 4 days and then sacrificed 9 days after withdrawal of the compound. Formation of spicules is almost normal. Toluidine blue stain ($\times 125$).

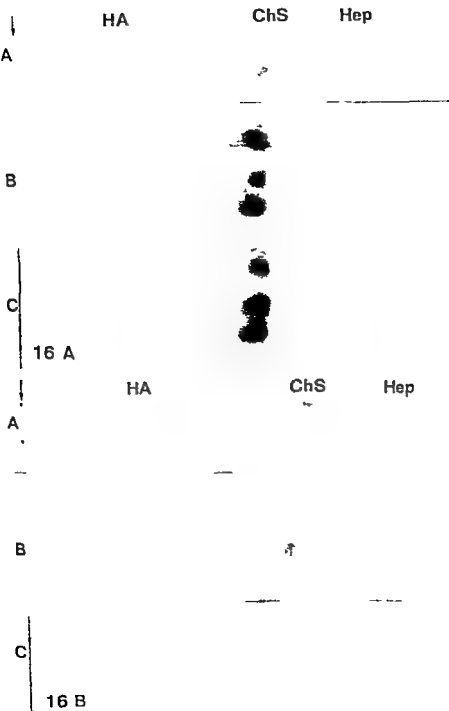




Figs 12 and 13

Fig 12 Cartilage metaphysis border of a lathyrotic rabbit given AAN through 4 days and then sacrificed 5 days after withdrawal of the compound. Formation of spicules is still completely disorganized. Toluidine blue stain ($\times 120$)

Fig 13 Cartilage metaphysis border of a lathyrotic rabbit given AAN through 4 days and then sacrificed 9 days after withdrawal of the compound. Formation of spicules is almost normal. Toluidine blue stain ($\times 125$)



HA

ChS Hep

C

17 A

HA

ChS

Hep

A

B

C

17 B

That electrophoresis should show the presence of chondroitin sulphate was expected (Dziewiatkowski 1957), but as the standard was not chemically well defined, the type of chondroitin sulphate found could not be identified. The presence of hyaluronic acid seems to be established. This concurs with the chemical demonstration of hyaluronic acid in epiphyseal cartilage (Hjertquist 1964). The finding of the same two types of mucopolysaccharides in cartilage and bone lends some support to the view that metaphyseal mucopolysaccharides may, at least in part, be derived from epiphyseal cartilage (Dziewiatkowski 1957). The appearance of a stronger hyaluronic acid band in lathyrtic metaphysis samples was reproducible, but should be regarded only as circumstantial evidence of an increase of this substance. Although scanning of the strips (Harcldmann & Brunish 1965) was also done, this procedure remained unreliable as quantitative assessment. The significance of the faint intermediate bands is uncertain, since they were not constant.

SUMMARY

Changes in epiphyseal cartilage and metaphyseal spongiosa of extremity growth zones of 2-3 week old rabbits rendered acutely lathyrtic with aminoacetonitrile are described, macro- as well as microscopically. Attention is particularly drawn to the disorganized endochondral ossification at the cartilage metaphysis border. Electrophoresis on cellulose acetate strips of alkali extracted mucopolysaccharides from epiphyseal cartilage and metaphyseal spongiosa indicates the presence of chondroitin sulphate and hyaluronic acid in both types of tissue.

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Fig 17

- a Metaphyseal border - control sample
b Metaphyseal border - lathyrtic sample

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MALIGNANT MELANOMAS OF THE EYE AS STUDIED WITH A SPECIFIC FLUORESCENCE METHOD

By

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Dopa, 3,4-dihydroxyphenylalanine, is regarded as an important intermediary product in the synthesis of melanin (14, 11, 12). In model experiments dopa condenses with formaldehyde to form a strongly fluorescent product (4). Normal melanocytes and cells of pigmented naevi and malignant melanomas display fluorescence (8, 6) after a special formaldehyde treatment (3). It was assumed that the observed fluorescence was caused by dopa (6). Support for this assumption was obtained from the recent demonstration of dopa in malignant melanomas (5). In regard to the origin in common with the melanin producing cells in the skin and in the choroid of the eye an investigation of the fluorescence characteristics of ocular melanomas was of obvious interest.

MATERIALS AND METHODS

The material comprises ocular melanomas from 11 patients: 8 women and 3 men aged 37 to 75 years. Immediately after enucleation about half of the tumours were quick frozen in propane cooled by liquid nitrogen, freeze dried, and then treated with formaldehyde according to the method of *Fulek & Hillarp* (3, 4, 7). Controls for autofluorescence were obtained by excluding the formaldehyde in the treatment. Sections were obtained at 6-8 μ and analysed in the fluorescence microscope (7). For ordinary light microscopy fixation was performed in formalin and the sections were stained in haematoxylin and eosin. Classification according to *Callender* (1) was based on examination of freeze dried as well as formalin fixed material.

RESULTS

Clinical data and microscopic findings are summarized in Table 1.

As can be seen, four of the eleven melanomas displayed cells with a specific fluorescence, whereas the remaining seven contained no such cells. Under the conditions of observation the fluorescence was in the yellow-green range and localized to the cytoplasm, the nuclei being dark. In two of the four tumours most of the cells were strongly fluorescent (Fig. 1), whereas in the other two the fluorescent cells

TABLE 1

Case	Sex	Age	Localization	Pigment content	Type according to Gaffender	Fluorescence
1	♀	75	Ciliary body	High	B, scattered areas with epitheloid cells	++
2	♀	67	Choroid	Varying	A, some B	0
3	♀	68	Choroid and ciliary body	Slight	B, very few epitheloid cells	0
4	♀	57	Choroid	None	B, many epitheloid cells	0
5	♂	55	Iris		A	0
6	♂	73	Choroid	None	B and epitheloid cells	0
7	♀	40	Choroid and ciliary body	Varying	B and scattered epitheloid cells	+++
8	♀	54	Choroid	Varying	A and B, scattered areas with epitheloid cells	+(+) or 0
9	♀	65		Varying	B	0
10	♀	37	Choroid and ciliary body	Varying	Epitheloid	+++
11	♂	40	Choroid and ciliary body	Slight	B	0

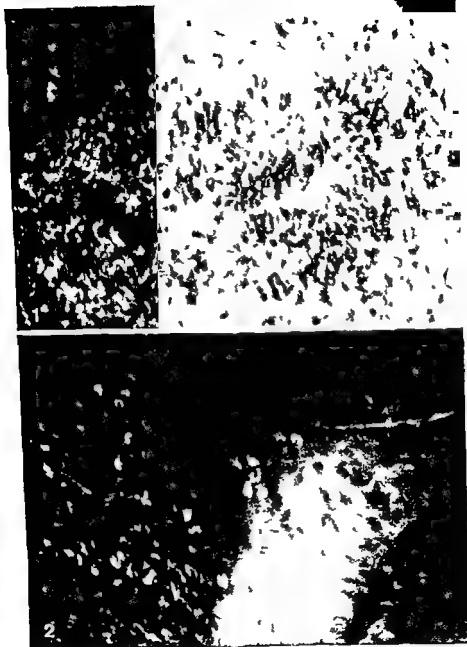


Fig. 1 Human ocular malignant melanoma cells showing strong specific fluorescence.

Fig. 2 Human ocular malignant melanoma cells showing moderate fluorescence.

Fig. 3 Human ocular malignant melanoma cells showing no specific fluorescence.

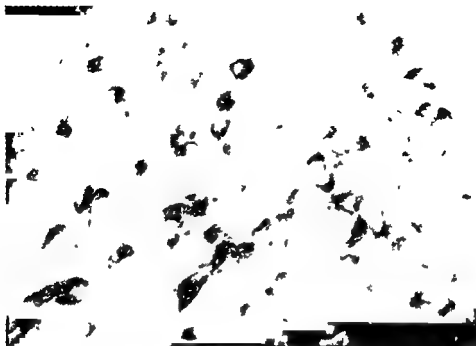


Fig 3

Human neular malignant melanoma. The cells with a specific fluorescence are polymorph with a dark nucleus and varying intensity of the cytoplasmic fluorescence $\times 530$

were few and had a lower fluorescence intensity (Fig 2). In the two tumours with a smaller number of fluorescent cells, regions containing non-fluorescent cells were irregularly distributed throughout the tumour. Usually the degree of pigmentation and the fluorescence seemed to vary independently of each other. However, to a certain degree the fluorescence could be masked by a dense accumulation of pigment. The morphology of the fluorescent cells varied with intermingled rounded, polygonal, spindle-shaped or dendritic cells (Fig 3). The fluorescence of the cells varied from none at all to very intense but did not seem to be correlated with any special cell type. However the cells with the most intense fluorescence were often rounded and large.

Comparison with routine stained sections showed that cells with the same appearance could be either fluorescent or non fluorescent.

In one patient with strongly fluorescent cells in the tumour, the branched melanocytes in the anterior layer of a part of the iris displayed yellowish fluorescence throughout the cytoplasm. The intensity of the fluorescence here varied between different cells and was generally highest in the most superficial cells.

DISCUSSION

Only four of the eleven malignant melanomas of the eye displayed fluorescent cells. In the skin all malignant melanomas studied contained fluorescent cells (6). This difference in the fluorescence characteristics of the tumours of the skin and the eye could not be predicted from earlier knowledge of these tumours, and must be considered significant.

There is evidence that tyrosinase activity is correlated with the fluorescence intensity of melanin producing cells (6). Thus, highly active melanin producing cells of the skin demonstrate tyrosinase activity (9, 10) and strong fluorescence (6) whereas no tyrosinase activity (13) or specific fluorescence has been found in the melanin containing cells of the adult eye (2).

The total absence of fluorescence in many tumours and the absence of fluorescence in many cells in some of the melanomas containing fluorescent cells shows that fluorescence is not firmly bound to the neoplastic growth of the melanin-producing cells. It seems possible that the melanin formation in some ocular melanomas need not result in high concentrations of dopa within the melanin synthesizing cell. Differences in fluorescence between cells in one and the same melanoma might be explained by the cells being different in melanin synthesizing capacity but might perhaps also be a sign of different timing of melanin synthesis in the cells.

The fluorescence of the cells could not be correlated with the pathological subdivision of ocular melanomas according to *Callender* (1) nor with the melanin content. The strongest fluorescence observed seemed to be in large epithelioid cells and in highly branched cells. In melanomas of mixed type the lack of correlation between fluorescence and the morphology of the cells was particularly evident.

In one patient with an ocular melanoma containing fluorescent tumour cells, fluorescing melanocytes were observed in a part of the iris. It seems possible that the tyrosinase of the observed iridic melanocytes was activated by the malignant growth of the eye. Activation of iris freckles has been observed in connection with ocular melanomas (15).

SUMMARY

Eleven ocular melanomas were studied with a specific fluorescence method demonstrating catecholamines and 5-hydroxytryptamine and their immediate precursors dopa and 5-hydroxytryptophan. In four tumours fluorescent cells were found. The intensity of fluorescence did not correlate with the cell type or with the haematoxylin eosin stained sections. The absence of fluorescence was observed in many ocular melanomas in contrast to the presence of fluorescence in cutaneous melanomas.

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EVOLUTIONARY TRENDS OF ANEUPLOID BLOOD CULTURE CELL POPULATIONS DURING PROGRESSION AND TREATMENT OF CHRONIC MYELOGENOUS LEUKAEMIA

By

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It appears probable from previous cytogenetic investigations that, within the Ph¹-positive cell populations, selection of certain cell categories occurs during progression and treatment with cytostatics of chronic myelogenous leukaemia. Both during progression of the disease and during therapy, hyperdiploid cells appear to be favoured at the expense of other Ph¹-positive cells (Pedersen 1966a). The high prevalences of hyperdiploid, Ph¹-positive cells in the late phase of the disease and during treatment with cytostatics may, however, be due to a few, well-represented cell clones. If this is the case, the clones concerned are possibly favoured but not necessarily hyperdiploid karyotypes as a whole. If the selective pressure of the leukaemic *in vivo* environment, including cytostatics, electively favours the proliferation and/or survival of hyperdiploid cells, increased prevalence of various hyperdiploid karyotype variants would be anticipated in the late phase of the disease and during treatment with cytostatics.

The object of the present work is to illustrate this question by comparing the number of aneuploid karyotype variants which have developed independently of one another in the phase and treatment groups of a blood culture material.

In order to assess the extent to which artificial factors, including cytostatics, are *eo ipso* responsible for the aneuploid state of the population, the Ph¹-positive cells will be compared with a Ph¹ negative population which originates from the same blood culture material as the Ph¹-positive cells.

MATERIAL AND METHODS

The material consists of Ph¹ positive and Ph¹ negative blood culture cells from patients with clinically and haematologically identical chronic myelogenous leukaemia.

were submitted to investigation on one to seven different occasions in the course of the disease. Further information concerning the collection of the material, its nature and extent, the technique of the *in vitro* culture, harvesting of the cultured material and preparation of the specimens for chromosome analysis is given elsewhere (Pedersen 1966 a) where the principles for selection of the mitoses for analysis and the method of analysis are also mentioned.

The aneuploid cells are subdivided according to the chromosome counts into the groups <45 , 45 , $46U$ and >46 . The $46U$ group consists of unbalanced diploid cells, i.e. cells with 46 chromosomes but aneuploid karyotypes (Levan & Müntzing 1963). The Ph^1 negative cell material originates as mentioned from the same cultures as the Ph^1 positive cells and was therefore, cultured, harvested and prepared in the same manner and exposed to the same cytostatic treatment as the Ph^1 positive cells. Further, Ph^1 positive and negative cells were analysed at random. The two cell populations were finally subdivided into groups according to the same principles. Cells which could not be classified with certainty as Ph^1 positive or Ph^1 negative were not included in the present material.

In order to illustrate the structure of the aneuploid cell population during the progression of the disease, the material is subdivided into three groups:

1. Cultures from untreated patients, all of whom were newly diagnosed at the time of investigation (untreated phase).

2. Cultures from treated patients in the early phase of the disease, i.e. patients who at the time of investigation had received cytostatic treatment for a total of less than 150 days.

3. Cultures from patients in the late phase of the disease, i.e. patients who at the time of investigation had received cytostatic treatment for 150 days or more.

In order to illustrate the possible effects of treatment, the material was further subdivided into two treatment groups:

1. Cultures from patients who at the time of investigation were receiving cytostatic treatment or whose last treatment series was concluded less than ten days previously (group T 10—).

2. Cultures from patients whose last treatment series was concluded more than ten days previously (group T 10+).

In each of the groups in the material, only one representative from each aneuploid karyotype from each patient is included. The prevalences of aneuploid cells calculated on this basis, thus express the frequencies with which the aneuploid karyotype variants have developed independently of one another.

The phase and treatment groups will be compared in the following manner: untreated/early phase, early/late phase and groups T 10+/T 10—. In each pair of groups compared, cultures are only included from patients represented in both groups. In the early and late phase groups, cultures from the same patient belong to the same treatment group. In the treatment groups, cultures from the same patient belong to the same phase group. The relevant material includes 65 cultures from 18 patients.

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RESULTS

The untreated and the early treated phases of the disease do not show significantly different prevalence of members in the various chromosome count groups (Table 1), neither in the Ph^1 -positive nor the Ph^1 -negative cell population. The late phase of the disease, on the other hand, contains significantly more Ph^1 -positive cells with hyperdiploid karyotypes than the early phase (Table 2). Further, the late phase shows a tendency to the lowest frequency of 45 cells and highest prevalence of 46U cells. In the Ph^1 -negative population, the two phases do not show any definitely different distribution in the aneuploid

TABLE 1

*Chromosome Count Distributions in Ph¹ Positive and Ph¹ Negative Cells
Untreated versus Early Treated Phase of Disease*

Chromosome Count	Ph ¹ Positive Cells			Ph ¹ Negative Cells		
	Phase of Disease		P	Phase of Disease		P
	Untreated	Early		Untreated	Early	
<45	13	8	>0.05	5	8	>0.05
45	17	9	>0.05	3	19	>0.05
46U	5	4	>0.05	—	5	>0.05
>46	1	5	>0.05	1	7	>0.05
Total	36	26	>0.05	9	36	>0.05
No of Cultures	8	13		7	10	
Analysed Cells	245	145		110	342	

TABLE 2

*Chromosome Count Distributions in Ph¹ Positive and Ph¹ Negative Cells
Early versus Late Phase of Disease*

Chromosome Count	Ph ¹ Positive Cells			Ph ¹ Negative Cells		
	Phase of Disease		P	Phase of Disease		P
	Early	Late		Early	Late	
<45	5	7	>0.05	2	2	>0.05
45	11	8	>0.05	7	6	>0.05
46U	5	18	>0.05	8	5	>0.05
>46	6	43	<0.0005	2	—	>0.05
Total	27	76	0.010-0.025	19	13	>0.05
No of Cultures	9	8		5	3	
Analysed Cells	169	287		185	66	

TABLE 3

*Chromosome Count Distributions in Ph¹ Positive and Ph¹ Negative Cells
Treatment Group T 10+ versus T 10—*

Chromosome Count	Ph ¹ Positive Cells			Ph ¹ Negative Cells		
	Treatment Groups		P	Treatment Groups		P
	T 10+	T 10—		T 10+	T 10—	
<45	8	3	>0.05	7	4	>0.05
45	15	5	0.05	16	12	>0.05
46U	12	10	0.05	16	20	>0.05
>46	14	23	>0.01	9	3	>0.05
Total	47	41	0	48	39	>0.05
No of Cultures	16	17		14	14	
Analysed Cells	301	19		319	332	

chromosome count groups. In Table 3, the two treatment groups are compared. The prevalence of hyperdiploid, Ph⁺-positive cells is significantly higher in group T 10— than in group T 10+. The frequency of Ph⁺ positive 45 cells is highest in the group T 10+ but the difference from group T 10—, however, is not significant. The Ph⁺ negative population does not show any definitely different prevalences.

TABLE 4
Chromosome Count Distributions in Ph⁺ Positive versus Ph⁺ Negative Cells of Untreated, Early and Late Phases of Disease

Chromosome Count	Phase of Disease								
	Untreated			Early			Late		
	Ph ⁺ Pos	Ph ⁺ Neg	P	Ph ⁺ Pos	Ph ⁺ Neg	P	Ph ⁺ Pos	Ph ⁺ Neg	P
<45	13	5	>0.05	5	2	>0.05	7	2	>0.05
45	17	3	>0.05	11	7	>0.05	8	6	0.03
46L	5	—	>0.05	5	8	>0.05	18	5	>0.05
>46	1	1	>0.05	6	2	>0.05	43	—	0.001, 0.005
Total	36	9	>0.05	27	19	>0.05	76	13	>0.05
No. of Cultures	8	7		9	5		8	3	
Analysed Cells	243	110		169	185		287	66	

TABLE 5
Chromosome Count Distributions in Ph⁺ Positive versus Ph⁺ Negative Cells of Treatment Groups T 10+ and T 10—

Chromosome Count	Treatment Groups					
	T 10+		P	T 10—		P
	Ph ⁺ Pos	Ph ⁺ Neg		Ph ⁺ Pos	Ph ⁺ Neg	
<45	6	2	>0.05	3	4	>0.05
45	15	16	>0.05	5	12	>0.05
46L	12	16	>0.05	10	20	>0.05
>46	14	9	>0.05	23	3	<0.0005
Total	47	43	>0.05	41	39	0.001, 0.010
No. of Cultures	16	14		17	14	
Analysed Cells	301	339		195	312	

In Table 4 the distributions of the Ph⁺ positive and negative cells in the four chromosome count groups in the three phase groups of the material are compared. Neither the untreated nor the early treated phases show definite differences in prevalence between the two populations. In the late phase, hyperdiploid cells occur significantly more frequently in the Ph⁺ positive than in the Ph⁺ negative population. In

Table 5 the two populations are compared in each treatment group *per se*. While group T 10+ does not show any definite difference, the Ph¹-positive prevalence of hyperdiploid cells is significantly higher than the Ph¹ negative in group T 10—

DISCUSSION

The cytogenetic basic material from which the present material originates is particularly heterogeneous. Attempts were made to make the phase and treatment groups comparable in the following ways

1 It cannot be excluded in advance that the tendency to form aneuploid cells is an individual feature, i.e. independent of the progression and treatment of the chronic myelogenous leukaemia. If the patients included in the phase and treatment groups compared are not identical, the possibility must be borne in mind that the differences between these groups may be due to the patients in the groups rather than the phase of the disease and the situation as regards treatment. An attempt was made to eliminate this possible source of error by only including patients who were represented by one or more cultures in both groups in each pair of groups compared. Further, it was required that corresponding cultures in the early and late phase groups must belong to the same treatment group and that the corresponding cultures in the treatment groups T 10+ and T 10— must originate from the same phase of the disease. It is thus possible to include both T 10— and T 10+ cultures in both the early and the late phase groups and to include cultures from both the early and late phase groups in both treatment groups T 10+ and T 10—. It should be noted, however, that the early phase group which is compared with cultures from untreated patients consists of both T 10— and T 10+ cultures.

■ By including only one representative of each aneuploid karyotype in each of the groups in the material, aneuploid clones comprising numerous cells are prevented from dominating the aneuploid cell populations of the corresponding phase and treatment groups.

These attempts to work with reasonably comparable groups have, however, resulted in considerable reduction of the extent of the relevant material.

The results of the present investigation are by and large, in agreement with a previous investigation of the same cytogenetic basic material (Pedersen 1966a). This investigation together with the present investigation shows that during progression of the disease, increasing prevalence of hyperdiploid, Ph¹ positive cell occurs and that these cells include an increasing number of karyotype variants. Further, the prevalence of hyperdiploid Ph¹ positive cell and the number of hyperdiploid karyotypes involved are significantly greater in the treatment group T 10— than in group T 10+. In comparing the cytogenetic profiles of the treatment groups it is, however, to be borne in mind that

the average duration of the cytostatic treatment was somewhat longer in group T 10— (235.5 days per Ph¹-positive cell) than in group T 10+ (183.11 days per Ph¹-positive cell). It does not appear probable that this difference can explain the divergent cytogenetic conditions in the two groups.

The prevalence of Ph¹ positive 45 cells was previously found to decrease during progression of the disease and during treatment with cytostatics, while the frequency of Ph¹-positive 46 U cells shows an indefinite relationship to progression of the disease and decreases during treatment (Pedersen 1966a). In the present material, the two categories of cells show tendencies which are in agreement with this but the differences are not significant.

In contrast to the Ph¹ positive cells, the composition of the aneuploid Ph¹-negative cell population does not reveal any definite dependence upon progression or treatment of the disease. Had cytostatic treatment been a material cause of development of aneuploid cell variants, it would be anticipated that the Ph¹-positive and -negative cell populations would show corresponding development during progression and treatment. As this is not the case, it may be concluded that cytostatics *co ipso* do not cause aneuploidy in blood culture cells from patients with chronic myelogenous leukaemia to any appreciable extent.

The high prevalence of hyperdiploid, Ph¹-positive karyotypes in the late phase group and in treatment group T 10— cannot be due to *in vitro* selection of hyperdiploid cells in the cultures which originate from blood samples with sporadic occurrence of such cells, as a comparison of the cytogenetic conditions in uncultured bone-marrow biopsies from patients with chronic myelogenous leukaemia and blood cultures which originate from the same patients and the same time did not show signs of selection of hyperdiploid cells (Pedersen 1966b).

The present observations are, however, consistent with the assumption that selection of hyperdiploid, Ph¹-positive cells occurs *in vivo* in the late phase of chronic myelogenous leukaemia. This selection is probably due not only to weakening of the resistance of the organism towards cytogenetically abnormal cells as this would presumably favour all categories of aneuploid cells, Ph¹-positive and Ph¹-negative. Bearing in mind that the highest prevalences of hyperdiploid Ph¹-positive cells (Pedersen 1966a) and karyotype variants (the present investigation) originate from cultures from patients receiving intensive cytostatic treatment, it appears reasonable to presume that the cytostatics employed are, to a great extent, responsible for selection of Ph¹-positive cells with hyperdiploid karyotypes because these are, somehow or other, less sensitive to the treatment than other cells.

The present investigation cannot illustrate the question whether hyperdiploid chromosome counts *co ipso* are associated with selective advantages or whether such advantages are associated with definite karyotypes.

SUMMARY

In a previous investigation, the prevalence of hyperdiploid, Ph^1 -positive cells in a blood culture material from 29 patients with chronic myelogenous leukaemia investigated cytogenetically was found to be significantly higher in the late phase of the disease than in its early phase and higher in cultures from patients receiving treatment with or recently treated with cytostatics than in cultures from patients in whom the last period of treatment had been concluded longer ago (Pedersen 1966a). The object of the present work was, in the same cytogenetic basic material, to investigate whether the number of hyperdiploid karyotype variants involved increases during the progression and treatment of the disease. For this reason, only one representative of each aneuploid karyotype in each of the phase and treatment groups was included in the material from each patient. In order to increase the comparability of the groups, only patients were included in each pair of groups compared who were represented by one or more cultures in both groups of the pair.

While cultures from untreated patients and treated patients in the early phase of the disease did not show definite differences, the prevalence of hyperdiploid, Ph^1 positive karyotype variants was significantly higher in the late phase of the disease than in its early phase and higher in cell populations from patients receiving cytostatics at the time of investigation or who had concluded this treatment a few days previously than in the population from patients whose last series of treatment had been concluded longer ago. The remaining aneuploid chromosome count groups did not show any significant difference between the phase and treatment groups compared.

As a comparable Ph^1 negative population did not show differences associated with progression and treatment of the disease, it is concluded that the differences observed in the Ph^1 positive cell populations cannot be due to direct karyotype damage caused by cytostatics to any appreciable extent, as the Ph^1 -positive and -negative populations have been subjected to cytostatics to much the same degree. The observations seem to indicate that the spectrum of viable hyperdiploid, Ph^1 -positive karyotypes is broader in the late than in the early phase of the disease and broader during cytostatic treatment than in treatment free periods. An important reason for this state of affairs may be that cells with hyperdiploid, Ph^1 -positive karyotypes are relatively more resistant to the cytostatics employed than other cells.

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CYTOGENETIC STRUCTURE OF ANEUPLOID BLOOD CULTURE CELL POPULATIONS DURING PROGRESSION AND TREATMENT OF CHRONIC MYELOGENOUS LEUKAEMIA

By

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Previous investigations of Ph^1 -positive blood culture cells from patients with chronic myelogenous leukaemia suggest that selection of cells with certain chromosome counts occurs at the expense of other cells during progression of the disease. Among other features, the cell populations from patients in the late phase of the disease appear to contain significantly higher prevalences of hyperdiploid, Ph^1 -positive cells than populations from patients in the early phase of the disease (Pedersen 1966a) and, similarly, the number of hyperdiploid, Ph^1 -positive clones was found to be significantly higher in the late than in the early phase (Pedersen 1967). In addition, the frequency of hyperdiploid, Ph^1 -positive cells was found to be definitely higher in blood cultures from patients who were receiving treatment with cytostatics at the time of investigation or in whom the first series of treatment had been concluded immediately prior to the cytogenetic investigation than in blood cultures from patients in whom treatment had been concluded longer ago. The former treatment group contained, further, a higher prevalence of different hyperdiploid, Ph^1 -positive clones than the latter group. It appears possible that selection of hyperdiploid, Ph^1 -positive cells, on a cytogenetic basis, depends upon their karyotypes rather than their chromosome counts. In the present investigation, an attempt will be made to illustrate this question by comparing the karyotype patterns in Ph^1 -positive, aneuploid karyotypes from different phase and treatment groups. The investigation was carried out on the same cytogenetic material as formed the basis for the works mentioned above. The Ph^1 -positive cell material was compared with a comparable Ph^1 -negative material in an attempt to estimate the possible significance of cytostatic drugs and technical factors for the Ph^1 -positive aneuploidy.

MATERIAL AND METHODS

The material consists of Ph^1 positive and negative blood culture cells from patients with clinically and haematologically typical chronic myelogenous leukaemia who were investigated on 17 different occasions during the course of the disease. Further information concerning collection of the material, its nature and extent, the technique of the *in vitro* culture, harvesting of the blood cultures and preparation of the specimens is given elsewhere (Pedersen 1966a) where the principles of selection of the mitoses in the material for analysis and the methods of analysis are described in more detail.

In many cells it is not possible to differentiate λ from the other small acrocentric chromosomes with certainty. This is particularly true in the case of Ph^1 positive cells where partially despiralized chromatin substance makes distinction difficult. For this reason group (21-22) and λ are treated as one group in the present work.

The object of the investigation is to determine the frequency with which members of the various chromosome groups are lost and acquired during the abnormal cell divisions which are the cause of the aneuploid clones in the material. As members of an aneuploid clone are probably all the result of one or the same abnormal cell divisions, only one representative of each aneuploid karyotype was included in each of the phase and treatment groups in the material of aneuploid cells; the chromosomal distribution of which has been published elsewhere (Pedersen 1967).

An attempt was made to illustrate the structure of the aneuploid cell population during progression of the disease by comparison of cells from three phases of the disease:

1. Cultures from untreated patients. All of the patients in this group were newly diagnosed.
2. Cultures from patients in the early phase of the disease: i.e. from patients who had been treated with cytostatics for less than a total of 100 days.
3. Cultures from patients in the late phase of the disease: i.e. from patients who

was in addition

receiving cytostatic treatment or in whom the last series of treatment was concluded less than ten days previously (group T 10—).

2. Cultures from patients whose last series of treatment had been concluded more than ten days previously (group T 10+).

Patients were only included in the pairs of phase and treatment groups compared if they were represented by one or more cultures in both of the groups of the pair concerned. In addition early and late phase cultures from the same patient originate from the same treatment group. Similarly, in the treatment groups cultures from the same patient belong to the same phase group. Despite this the average duration of the cytostatic treatment was somewhat longer in group T 10— (235.5 days per Ph^1 positive cell) than in group T 10+ (183.6 days per Ph^1 positive cell). After sorting out in this manner the relevant material comprises 65 cultures from 11 patients.

The Ph^1 negative cell material originates from the same cultures as the Ph^1 positive cells. Both cell populations were therefore grown, harvested and prepared in the same manner and were exposed to cytostatic treatment to the same or more or less the same extent.

material thus does not contain any cultures which lack small acrocentric chromosomes; the prevalence of λ members in Ph^1 negative cells could not be calculated.

The prevalence of extra chromosomes divided by the number of chromosomes observed in the group is the number of chromosomes observed in the group. The prevalence of extra chromosomes divided by the number of chromosomes observed in the group is the number of chromosomes observed in the group.

TABLE 1
Prevalences of Extra Chromosomes in Phi Positive and Phi Negative Cells
Untreated versus Early Treated Phase of Disease

Chromosomal Groups	Phi Pos Cells		P	Phi Neg Cells		P
	Untreated Extra/Others	Phase of Disease Early Treated Extra/Others		Untreated Extra/Others	Phase of Disease Early, Treated Extra/Others	
21 22 X	6/1136	5/644	>0.05	0/503	3/1657	>0.05
19 20	2/974	1/579	>0.05	0/438	3/1358	>0.05
17 18	0/977	0/576	>0.05	0/439	3/1369	>0.05
10	0/484	0/289	>0.05	0/218	1/2040	>0.05
13 15	0/1463	2/986	>0.05	0/657	4/5153	>0.05
6 X 12	1/3749	2/2246	>0.05	1/1692	2/3404	>0.05
1 5	0/2449	0/1449	>0.05	0/1100		
Total	9/11232	10/6769	>0.05	1/5047	17/15658	>0.05
No of Cultures	8	13	7	10		

TABLE 2
*Pretalences of Extra Chromosomes in Ph1 Positive and Ph1 Negative Cells
 Early versus Late Phase of Disease*

Chromosome Groups	Ph1 Pos Cells Phase of Disease		P	Ph1 Neg Cells Phase of Disease		P
	Early Extra/Others	Late Extra/Others		Early Extra/Others	Late Extra/Others	
21	3/789	52/1419	<0.0005	6/779	0/273	>0.05
13	3/671	13/1141	>0.05	0/738	0/262	>0.05
17	1/678	1/1036	>0.05	2/738	3/232	>0.05
10	1/337	0/571	>0.05	3/338	0/131	>0.05
1	1/1009	8/1713	>0.05	0/1103	0/396	>0.05
12	4/2578	150/4304	<0.0005	4/2514	2/1042	>0.05
5	0/1689	24/2864	<0.0005	1/1846	1/657	>0.05
Total	13/7748	248/13108	<0.0005	16/8480	6/3023	>0.05
No of Cultures	9	8	5	3	3	

calculated by dividing the number of absent chromosomes by the number of members observed in the chromosome group concerned. Cells with 43 or fewer chromosomes are not included in these calculations.

The basis for the calculations of prevalence of extra (21-22-1) members in the Ph¹ negative cells differed slightly from the basis for the other calculations. As the number of absent (21-22-1) members in the Ph¹ negative cells is not known and therefore cannot be included in the calculation, the number of small acrocentric chromosomes recorded is slightly too high in relation to the number of extra members. The prevalences of extra (21-22-1) members calculated in Ph¹ negative cells is, therefore, slightly lower than the actual number.

The frequencies of extra and absent chromosomes in the phase and treatment groups of the material were compared by means of χ^2 tests. Where the expected numbers were less than 5, the probability for the distribution observed in the groups compared was found by means of tables of binomial probability distributions.

RESULTS

The frequency of extra members in each individual chromosome group is compared in Table 1 in cultures from the untreated and the early treated phases of the disease. Neither the Ph¹-positive nor -negative population show significant differences. In the late, Ph¹-positive phase group, extra (21-22-Y), (6-X-12) and (1-5) were significantly more frequent than in the corresponding early phase group (Table 2). The extra (1-5) members originate from two cultures from two patients. All 24 extra chromosomes belong to chromosome pair no 1. The Ph¹-negative population does not show any definite difference between the two phase groups. Table 3 shows that extra (6-X-12) occur more frequently in the Ph¹-positive group T 10— than the group T 10+, whereas the Ph¹ negative cells do not show significant differences between the two treatment groups.

Table 4 compares the prevalences of absent members within the individual chromosome groups in cultures from the untreated and early treated phase groups. No significant differences were found in either the Ph¹-positive or the Ph¹-negative populations. Members of the (17-18) group were absent in Ph¹-positive cells more frequently in the late phase of the disease than the early phase (Table 5). The Ph¹-negative population does not show any significant difference between the two phase groups. Neither in the Ph¹-positive nor in the Ph¹-negative populations were significant differences in prevalence of absent chromosomes observed between the two treatment groups (Table 6) but the Ph¹-positive cells, however, showed a tendency to the greatest prevalence of absent (17-18) members in group T 10— ($0.05 < P < 0.10$).

Comparison of Ph¹-positive and Ph¹-negative prevalences of extra and absent chromosomes in each individual chromosome group in the phase and treatment groups of the material gives results which, by and large, are in agreement with the above. Cultures from the untreated and early treated phases do not show definite differences. In the late phase, extra (21-22-Y), extra (6-X-12) and absent (17-18) members were significantly most frequent in the Ph¹-positive population (0.001

TABLE 3
Prevalences of Extra Chromosomes in Ph1 Positive and Ph1 Negative Cells
Treatment Group T 10+ versus T 10—

Chromosome Groups	Ph1 Pos Cells		P	Ph1 Neg Cells		P
	T 10+ Extra/Others	Treatment Groups T 10— Extra/Others		T 10+ Extra/Others	Treatment Groups T 10— Extra/Others	
21	0/1393	5/953	>0.05	0/1535	0/1019	>0.05
11-20	0/1200	5/779	>0.05	3/1309	2/1301	>0.05
1-19	0/1193	1/765	>0.05	2/1307	1/1302	>0.05
11	3/33	0/187	>0.05	1/670	0/652	>0.05
1	1/1757	4/1167	>0.05	3/2039	2/1957	>0.05
1 X	11/4012	19/2970	<0.0005	11/5191	10/4912	>0.05
1	0/3005	0/1148	>0.05	0/1395	3/9201	>0.05
Total	11/13802	64/8135	<0.0005	30/15624	24/15008	>0.05
No. of Cultures	16	16		14	14	

TABLE 4
*Prevalence of Absent Chromosomes in Pht Positive and Pht Negative Cells
 Untreated versus Early Treated Phase of Disease*

Chromosome Groups	Pht Pos Cells Phase of Disease		P	Pht Neg Cells Phase of Disease		P
	Untreated Absent/Others	Early, Treated Absent/Others		Untreated Absent/Others	Early, Treated Absent/Others	
21 22 X	5/1142	6/649	>0.05	2/428	6/1361	>0.05
19 20	6/976	1/580	>0.05	1/439	3/1372	>0.05
17 18	1/977	4/576	>0.05	2/218	5/678	>0.05
16	6/484	1/289	>0.05	1/657	7/2041	>0.05
19 15	7/1403	4/988	>0.05	5/1693	10/5157	>0.05
6 X 12	10/3750	4/2248	>0.05	0/1100	6/3406	>0.05
1 5	1/2442	1/1449	>0.05			
Total	38/11241	21/6779	>0.05	13/4545	36/14015	>0.05
No. of Cultures	8	13		7	10	

TABLE 5
*Prevalences of Aberrant Chromosomes in Ph1 Positive and Ph1 Negative Cells
 Early versus Late Phase of Disease*

Chromosome Groups	Ph1 Pos Cells Phase of Disease		P	Ph1 Neg Cells Phase of Disease		P
	Early Absent/Others	Late Absent/Others		Early Absent/Others	Late Absent/Others	
21-22	7/792	7/1471	>0.05	2/718	2/262	>0.05
1-20	5/674	7/2154	>0.05	2/740	2/265	>0.05
1-18	1/676	52/1097	<0.0005	2/471	1/131	>0.05
11	1/338	3/571	>0.05	7/1103	6/396	>0.05
1-2	7/1010	9/1722	>0.05	7/2918	5/1044	>0.05
1-5	6/2582	10/4454	>0.05	4/1847	3/658	>0.05
	1/1789	6/2888	>0.05			
Total	26/7701	94/13356	<0.0005	24/7717	14/2756	>0.05
No of Cultures	9	8		8	7	

TABLE 6
Prevalences of Aberrant Chromosomes in Ph1 Positive and Ph1 Negative Cells
Treatment Group T 10+ versus T 10-

Chromosome Groups	Ph1 Pos Cells Treatment Groups		P	Ph1-Neg Cells Treatment Groups		P
	T 10+ Absent/Others	T 10- Absent/Others		T 10+ Absent/Others	T 10- Absent/Others	
21 22 X	9/1399	10 958	>0.05	3/1372	5/1305	>0.05
19 20	4/1206	1/784		5/1369	6/1303	
17 19	11/1193	15/766		4/679	2/652	
16	5/702	1/387		11/2032	5/1959	
13 15	9/1800	7/1171		12/5206	9/4922	
6 X 12	6/443	1/2955	>0.05	5/1395	7/3266	>0.05
1 X	2/1003	2/1948				
Total	46 1351	35/8999	>0.05	40/14053	34/13407	>0.05
No. of Cultures	16	16		14	14	

$< P < 0.005$, $P < 0.0005$, $0.005 < P < 0.010$ respectively.) In addition the Ph^1 positive population contains the highest prevalence of extra (6-12) members in both groups T10+ and T10- ($0.001 < P < 0.005$ and $P < 0.0005$ respectively.) Finally the Ph^1 positive prevalence of absent (17-18) members in group T10- is higher than the Ph^1 negative ($0.001 < P < 0.005$).

DISCUSSION

The phase and treatment groups compared are considered to be reasonably comparable. This point has been discussed elsewhere (Pedersen 1966b).

As mentioned previously in the present investigation no differentiation was undertaken between (21-22) and 1 in the group of small acrocentric chromosomes. As this group represents a considerable fraction of the extra chromosomes in the material it would be of interest to know whether the extra members were (21-22) or 1. Numerous aneuploid Ph^1 positive clones with extra small acrocentric chromosomes have been described in the literature (Adams *et al* 1961, Court Brown & Tough 1963, Hammouda 1963, Hammouda *et al* 1964, Kemp *et al* 1964, Lejeune *et al* 1965, de Grouchy *et al* 1965a and b, Kiosoglou *et al* 1965, Dougan & Woodliff 1965). None of these clones contained extra 1 chromosomes. The present material contains in the early phase three and in the late phase 52 extra small acrocentric chromosomes (Table 2). Of these 55 chromosomes 48 originate from cells with two Ph^1 . Despite the indefinite character of the chromatin in many of the cells the extra members with quite a great degree of certainty were found to be (21-22) in all 38 cells representing the 55 chromosomes. Extra 1 members do not appear to be included in the composition of the aneuploid Ph^1 positive cells in the material.

Analysis of the distribution of the chromosome counts in the aneuploid cells of the material revealed that in the Ph^1 negative population no dependence upon the total duration or the situation as regards treatment at the time of investigation was present (Pedersen 1967). The results presented here show that the pattern of extra and absent chromosomes in the aneuploid Ph^1 negative population similarly is uninfluenced by these factors. If cytostatic and/or technical factors were of decisive or material significance as aneuploidy producing factors the Ph^1 positive and the Ph^1 negative populations would have reflected this effect to a more or less uniform extent. As the prevalences of extra and absent chromosomes are much higher and differently distributed in the chromosome complement of the Ph^1 positive than in the Ph^1 negative population the Ph^1 positive aneuploids must have other causes.

The karyotype pattern of the Ph^1 positive cell population cannot be explained by *in vitro*

Ph^1 positive cell population in blood cultures from patients

with chronic myelogenous leukaemia do not appear to favour Ph^1 -positive cells with definite karyotypes (Pedersen 1966b).

The distribution of the chromosome counts in the aneuploid cells of the present material shows that cultures from patients in the late phase of the disease contain significantly higher prevalences of hyperdiploid, Ph^1 -positive clones than cultures from the early phase. In addition, these clones occur with greater frequency in treatment group T 10— than in group T 10+ (Pedersen 1967). It is apparent from the present investigation that the late phase and treatment group T 10— present higher Ph^1 -positive prevalences of extra (21-22), (6-X-12), (1-5) and absent (17-18) than the other groups of the material. The high prevalences of these karyotype abnormalities in the phase and treatment group which contains the greatest frequency of hyperdiploid, Ph^1 -positive cells suggests that these, particularly, characterize the hyperdiploid, Ph^1 -positive cell population. If selection of hyperdiploid, Ph^1 -positive cells was due to an abnormal chromosome count, prevalences of extra chromosomes would be anticipated to be more or less uniform in the various chromosome groups. Concentration of the extra chromosomes in three out of the seven chromosome groups suggests that selection of hyperdiploid, Ph^1 -positive clones in the leukaemic *in vivo* environment is due to the karyotypes of the clones and not to their chromosome counts.

It should be noted that the results of the present investigation must be assessed bearing in mind the limited extent of the material. The results must not be interpreted to suggest that, generally, selection of clones with extra (21-22), (6-X-12), (1-5) and absent (17-18) members occurs in patients with chronic myelogenous leukaemia. It is probable that other materials can demonstrate selection of clones with other karyotypes. For example, the Ph^1 -positive cells of this material contain only two extra (17-18) members (Table 2) as compared with 53 absent members of this group (Table 5) which gives the impression that Ph^1 -positive clones with extra (17-18) members are more poorly adapted to the leukaemic *in vivo* environment than clones in which (17-18) members are absent. The numerous descriptions in the literature of Ph^1 -positive, aneuploid clones with extra (17-18) members (Kemp *et al* 1963, Court Brown & Tough 1963, Hammouda *et al* 1964, Lejeune *et al* 1965, de Grouchy *et al* 1965 a and b) and few descriptions of clones in which members of this group are absent (Court Brown & Tough 1963, Hammouda 1963) give however, a different impression of the relative frequency of extra and absent (17-18) members in aneuploid, Ph^1 -positive clones. The clones described in the literature, however, as in the present results, are dominated by karyotypes with extra (21-22 Ph^1) (Adams *et al* 1961, Court Brown & Tough 1963, Hammouda 1963, Jung *et al* 1963, Reisman & Trujillo 1963, Trujillo & Ohno 1963, Hammouda *et al* 1964, Kemp *et al* 1964, Lejeune *et al* 1965, de Grouchy *et al* 1965 a and b, Kiosoglou *et al* 1965, Dougan &

Woodliff 1965, Erkman *et al* 1966) and extra (6-12) members (Ford & Clarke 1963, Court Brown & Tough 1963, Kemp *et al* 1963, Levan *et al* 1963, Hammouda *et al* 1964, Goh *et al* 1964, de Grouchy *et al* 1965 a and b, Lejeune *et al* 1965, Erkman *et al* 1966)

SUMMARY

In a cell material from blood cultures from patients with chronic myelogenous leukaemia investigated cytogenetically and grouped according to phase of the disease and situation as regards treatment at the time of investigation evidence was found previously that selection of hyperdiploid, Ph¹ positive cells occurs during progression and treatment of the disease (Pedersen 1967). In the present investigation, the patterns of extra and absent chromosomes of these groups are compared to illustrate the question of whether the selection mentioned above occurs on the basis of the chromosome counts of the clones or is dependent of their karyotypes.

Each pair of phase and treatment groups in the material includes only patients represented by one or more cultures in both of the groups of the pair. The material totals 65 cultures from 18 patients. Only one representative of each aneuploid karyotype from each patient is included in each group of the material. The numbers of extra and absent members are compared with the number of other chromosomes observed in each chromosome group after which the prevalences are compared in the groups of the material. A comparable Ph¹-negative cell material was compared with the Ph¹ positive material in order to evaluate the influence of iatrogenic and technical factors upon the cytogenetic conditions of the Ph¹ positive population.

In cultures from patients in the late phase of the disease the prevalences of extra (21-22), (6-12) and (1-5) members in Ph¹ positive cells were found to be significantly higher than in cultures from the early phase of the disease. In cultures from patients receiving treatment with cytostatics or who had recently received such treatment at the time of investigation the prevalence of extra (6-12) was significantly higher, and there was a tendency towards greater frequency of absent (17-18) members than in cultures from patients in whom the last series of treatments had been concluded longer ago.

As the Ph¹ negative population does not show corresponding conditions it is improbable that iatrogenic or technical factors are the cause of the development demonstrated. As selection of Ph¹ positive cells with certain karyotypes does not appear to occur in blood cultures (Pedersen 1966b), the results of the present investigation is presumably not due to *in vitro* favouring of Ph¹ positive cells with the chromosome abnormalities mentioned but only to *in vivo* selection of such cells. The results of the present investigation thus suggest that selection of hyperdiploid Ph¹ clones during progression and

treatment of chronic myelogenous leukaemia is primarily due to their karyotypes and not the high chromosome count *co ipso*

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ON THE SPECIFICITY OF HUMAN THYROGLOBULIN AUTO ANTIBODIES

By
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The induction of thyroglobulin auto antibodies seems to require a termination of immune tolerance towards thyroglobulin (8). In rabbits this tolerance may be terminated by injections of structurally altered homologous thyroglobulin (22).

Several other types of human auto-antibodies such as anti γ globulin factors and conglutinin seem to react with "hidden" antigenic determinants which are unmasked in immune aggregates (9, 2).

Both these lines of observation emphasize the rôle of structural changes of auto antigens as one mechanism behind auto-antibody formation. In the present investigation, attempts were made to detect human auto-antibodies against altered thyroglobulin molecules.

MATERIALS AND METHODS

Antigens. Thyroglobulin was prepared from normal or diseased human thyroids (hog and bovine thyroids by $(\text{NH}_4)_2\text{SO}_4$ fractionation between 37 per cent and 49 per cent saturation).

It was purified by Roitt & co's method of purity rabbit serum, 100 per cent saturation stored frozen at -20°C .

Some preparations of human thyroglobulin were lyophilized before they were stored at -20°C .

Fresh extracts of normal human thyroids were prepared from the thyroid of patients suffering from laryngeal carcinomas with no history of thyroid disease. Immediately after removal the thyroid tissue was minced gently by scissors mixed with 2 volumes (W/V) of sterilized saline and shaken gently at 4°C for 4 hours. The supernatant after centrifugation ($4000 \times g \times 30$ minutes) was if not otherwise stated used the same day for inhibition of hemagglutination.

Sera. The sera employed could be divided into 3 groups:

1. Sera from patients suffering from thyroid disease (mainly thyroiditis with high titers ($\geq 1:2500$ by the indirect hemagglutination method) of thyroglobulin auto-antibodies).

2. Sera of patients without thyroid disease and of healthy persons with high titers ($\geq 1:2500$) of thyroglobulin auto antibodies.

3. Sera from patients surgically treated for thyroid disease from whom thyroid tissue was obtained.

Hemagglutination. The indirect hemagglutination procedure according to Jensen's method (1) was employed. Human erythrocytes were formalinized sensitized as previously described (3, 6). The concentration of antigen used for

sensitization was 0.5 mg per ml against 1.2×10^8 erythrocytes per ml. Such cells have been calculated to contain about 35000 antigen molecules per cell (7).

Hemagglutination inhibition. Inhibition of cell hemagglutination was performed by addition of thyroglobulin or thyroid extracts immediately prior to addition of sensitized erythrocytes to serum dilutions in perspective. The reaction was read after incubation at 4°C over night.

Cell precipitation. The method of Ouchterlony (15) was performed as described by Fritsken (4) employing 0.03 M Earle's buffer at pH 8.6 with 0.03 per cent methyl orange, 1 per cent Difco Special Agar Noble and 0.1 per cent phenol.

Estimation of protein. Protein concentration was determined by the biuret method as described by Kabat & Mayer (11).

RESULTS

Inhibition of Hemagglutination of Cells Sensitized with $(\text{NH}_4)_2\text{SO}_4$ Fractionated and Lyophilized Thyroglobulin by Fresh Extracts of Normal Thyroids

Milgrom & Witebsky (13) have shown that structural changes are produced in rabbit γ globulins by $(\text{NH}_4)_2\text{SO}_4$ fractionation. Shulman & Armenia (19) demonstrated that thyroglobulin is partially denatured by lyophilization. In the present study attempts were made to detect auto-antibodies to fractionated and lyophilized thyroglobulin not reacting with native thyroglobulin.

For this purpose 31 sera were tested against erythrocytes sensitized with fractionated and lyophilized thyroglobulin. Sixteen sera were obtained from individuals without evidence of thyroid disease, 14 from patients suffering from thyroid disease, and the last serum was obtained from a patient (I F) who has previously been presented (8). This patient developed thyroglobulin antibodies to a titer of 1:2500 within a period of 6 months. As inhibiting antigen was used 0.01-0.02 ml per basin of a fresh thyroid extract from a normal thyroid containing approximately 40 mg protein per ml. This hemagglutination inhibition procedure was performed twice with thyroid extracts of different patients. On both occasions agglutination could be eliminated ($< 1:25$) from all sera tested. Similar extracts prepared from bovine thyroids did not interfere with agglutination.

Attempts to Demonstrate Antigenic Determinants Specific to the Thyroid of a Patient with Hashimoto's Thyroiditis

Six patients suffering from thyroiditis or myxoedema (J B, S A, A M, H V, L B, J S) with a positive reaction by gel precipitation were tested against an extract of the thyroid of a histologically verified Hashimoto patient (I I G) and an extract of a normal gland in adjacent walls. All sera gave rise to identity reactions between these 2 thyroid extracts as demonstrated by serum S A in Fig. 1.

Furthermore the same 31 sera used in the previous hemagglutination inhibition experiment were tested against erythrocytes coated with thyroglobulin prepared from the biopsy specimen of the Hashimoto

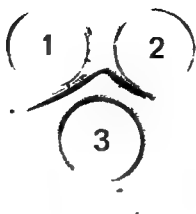


Fig. 1

Precipitation in gel by serum S A (3) extract of a normal thyroid (1) and an extract of thyroid tissue from a case of Hashimoto's disease (2)

thyroid Agglutination of these cells which revealed the same sensitivity as cells treated with ordinary thyroglobulin could be eliminated ($< 1:20$) by a fresh extract of a normal gland

Inhibition of Agglutination of Erythrocytes Sensitized with Autologous Thyroglobulin by an Extract of a Normal Thyroid

From six patients with thyroid auto antibodies autologous thyroglobulin was prepared from thyroid tissue removed by surgical treatment. Four of these patients suffered from thyrotoxicosis, one from a simple colloid struma and the last one from Hashimoto's thyroiditis. As shown in Table 1 cells coated with autologous thyroglobulin revealed the same sensitivity as cells coated with ordinary thyroglobulin and agglutination could be eliminated by an extract of a normal thyroid. This extract had been kept at 4°C for 14 days before the experiment was performed.

Cross Reactions of Human Auto anti- and Heterologous Thyroglobulins

Human thyroglobulin auto antibodies have been shown not to cross react with heterologous thyroglobulin by precipitation techniques (16). Using the method of indirect hemagglutination however cross reactions to a limited extent have been observed by Rose & Witebsky (18).

In the present study 2 out of 7 sera (S A and A M) from patients

with myxoedema or thyroiditis give rise to extensive cross reactions as shown in Table 2. On the other hand serum HA and LA revealed no cross reacting antibodies. The other 4 sera showed limited degrees of cross reactions. This variability from one serum to the other is in agreement with the observations of Rose & Witebsky (18). The clinical significance of this variability at present remains unknown.

TABLE 1

Agglutination of Erythrocytes Coated with Autologous Thyroglobulin and Hemagglutination Inhibition by an Extract of a Normal Thyroid

Patient	Clinical diagnosis	Titer of agglutination		Hemagglutination inhibition	
		with autologous thyroglobulin	with homologous thyroglobulin	by human thyroid extract (0.01 ml/cup)	by bovine thyroid extract (0.01 ml/ml)
PH	Thyrotoxicosis	1:2500	1:2500	<1:5	1:25
JO	"	1:25	1:25	<1:5	1:25
IM	"	1:25	1:25	<1:5	1:25
JH	"	1:5	1:5	<1:5	1:5
GS	Non-toxic diffuse Hashimoto's thyroiditis	1:25	1:25	<1:5	1:25
IG	"	1:25	1:25	<1:5	1:25

TABLE 2

Cross Reactions of Human Auto-antibodies with Heterologous Thyroglobulins

Patient	Clinical diagnosis	Hemagglutination titer of cells coated with thyroglobulin of				
		Man	Ox	Hg	Rat	Swine
JB	Irradiation myxoedema	1:25000		1:25	1:250	
SA	Thyroiditis	1:250000	1:200	1:25000	1:25000	1:200
AM	Thyroiditis	1:250000	1:50	1:2500	1:250	1:50
HA	Primary myxoedema	1:25000				
LN	Thyroiditis	1:25000				
IR	Thyroiditis	1:25000		1:10		
FS	Thyroiditis	1:250000		1:20	1:250	

— <1:25

Studies of rheumatoid factors by Vilgrom & Tonder (12) have indicated that denaturation of autologous γ globulins may unmask hidden determinants which resemble antigenic determinants on the surface of heterologous γ globulins. In order to establish whether cross reacting anti-thyroglobulins also were directed against such determinants, a hemagglutination inhibition assay was performed as shown in Table 3. The 2 strongest cross reacting sera (SA and AM)

were tested in dilutions of 1:25 against heterologous and homologous thyroglobulins, the ability to inhibit these reactions by various types of thyroglobulins was studied. As homologous antigens were used both fractionated thyroglobulin from pooled diseased thyroids and an extract of a normal thyroid prepared 14 days before the present experiment.

TABLE 3

Hemagglutination Inhibition of Cells Coated with Various Kinds of Thyroglobulin by Homologous and Heterologous Thyroglobulin

Serum	Thyroglobulin used for inhibition of agglutination						Cells coated with thyroglobulin from
	Human	Extract of normal human thyroid	Hovine	Hog	Rabbit	Guinea pig	
A VI tested in a dilution of 1:25	—*	—	+§	+	+	+	Man
	—	—	—	+	—	+	Ox
	—	—	+	—	+	+	Hog
	—	—	+	+	—	+	Rabbit
	—	—	+	+	—	—	Guinea pig
S A tested in a dilution of 1:25	—	—	+	+	+	+	Man
	—	—	—	+	—	+	Ox
	—	—	+	—	+	+	Hog
	—	—	+	+	—	+	Rabbit
	—	—	+	+	—	—	Guinea pig

— = agglutination inhibited

§ + = agglutination not inhibited

The hemagglutination inhibition studies show that all the cross reacting antibodies may be removed by absorption with homologous thyroglobulins. In contrast heterologous thyroglobulins in general only inhibit cells coated with the same type of thyroglobulin. An exception is made by rabbit thyroglobulin which in addition inhibited cells coated with bovine and guinea pig thyroglobulin.

Of the other sera only the cross reactions between J II and F S and rabbit thyroglobulin were studied by hemagglutination inhibition. These reactions likewise were inhibited by the extract of the normal thyroid.

DISCUSSION

The present studies have failed to detect populations of antibodies against autologous thyroglobulin homologous (NH₂)-SO₂ fractionated and lyophilized thyroglobulin thyroglobulin from a Hashimoto diseased thyroid or heterologous thyroglobulin which do not react with an extract of a normal thyroid.

One explanation of the negative findings could be that even a gentle preparation of a thyroid extract may unmask antigenic determinants against which auto antibodies are directed. Since components

diseased autologous thyroids or the thyroid of a patient with Hashimoto's disease could be detected

Sera from patients with thyroiditis or myxoedema gave rise to a variable degree of cross-reactions with heterologous thyroglobulins. Hog and rabbit thyroglobulin coated erythrocytes were found to be agglutinated more frequently and gave rise to higher titers than cells coated with guinea pig and bovine thyroglobulin.

The method of hemagglutination inhibition indicated that the cross reacting antibodies represented separate populations of antibodies. It is estimated that human auto-antibodies may at least react with 5 antigenic determinants of different specificity, all of which are present on "native" thyroglobulin molecules.

The present studies have failed to demonstrate human auto antibodies reacting with individual specific determinants or determinants specific to structurally altered thyroglobulin molecules.

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EVIDENCE FOR NON-RIBOSOMAL LOCATION OF STREPTOMYCIN RESISTANCE IN *ESCHERICHIA COLI* CARRYING THE MU-FACTOR

By

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The presence of the Mu-factor in cells of *Escherichia coli* furnishes these cells with two possible levels of protection against the lethal effect of streptomycin. When the Mu-factor is carried in its extra-chromosomal state (11) the cells have a low level resistance to streptomycin (9), the actual concentration of streptomycin tolerated depends upon the physiological state of the cells. In 10^{-4} of the cells in such a population the chromosome is modified, possibly by an attachment of the Mu factor to the bacterial chromosome, resulting in a significantly higher level of streptomycin resistance (11, 8). Experiments on uptake of ^{14}C -streptomycin (10) indicate that the low-level streptomycin resistance may be due to a decreased permeability of the drug. The higher level of resistance, however, could not be explained exclusively by a lack of penetration. The uptake of streptomycin found in these cells seemed higher, rather than lower, than in the low-level resistant cells, suggesting the possibility of an additional mechanism of streptomycin resistance in these cells.

The mechanism of true, one-step streptomycin resistance has been shown by several investigators to be due to an alteration in the structure of the ribosomes, rendering them insensitive to the interfering action of streptomycin (4, 5, 6, 1, 2). Rosenkrantz (16) has shown that ribosomes from cells of *E. coli* carrying the "multiple drug resistance" factor are inhibited by streptomycin to the same extent as ribosomes from ordinary streptomycin sensitive cells, suggesting another mechanism of streptomycin resistance in these cells. The author proposes a lack of penetration.

If the low level streptomycin resistance in the cells carrying the Mu-factor in its extrachromosomal state is solely due to a lack of penetration, one should expect to find their ribosomes as sensitive to streptomycin as are ordinary, sensitive ribosomes. The high level resistance, however, seems to depend upon an additional mechanism of protection. Could this mechanism be an alteration of the ribosomes? The present study was carried out in order to elucidate this question.

MATERIALS AND METHODS

Bacterial strains K12 T71 T71 str r T71Mu T71Mu str r and T71Mu thr his str r have formerly been described (11, 8). The bacteria were grown in Heart Infusion Broth (Difco).

Preparation of cell extracts Cell extracts (S-30) were prepared essentially as described by Nirenberg & Matthaei (13). The washed cells suspended in twice their wet weight of standard buffer (13) were disrupted by treatment at 15 amp twice for 50 seconds in an MSE ultrasonic disintegrator (60 watts) (20). Before this treatment the cell suspension was cooled to 0°C and during the treatment it was

* peptide The
d into peptide
(pH 7.8) 100
polyethanol 6
ate 8 μ moles
10 m μ moles

(0.5 μ C). Controls lacking poly U were always carried out simultaneously. All values were corrected for a non incubated control reaction terminated at zero time. The reaction was run at 37°C. Streptomycin when used was added at zero time. Poly U was added 20 minutes later. The reaction was terminated at 45 min by the addition of two volumes of 10 per cent trichloroacetic acid containing 1 mg of 1-phenylalanine per ml. The tubes were then heated for 10 min at 90°C, cooled, filtered and washed with 5 per cent trichloroacetic acid on Millipore filters (0.45 μ m pore size) mounted on aluminium planchets and counted on a Frieseke and Hoepfner windowless gas flow counter.

Orthomethyl and radioactive materials Disodium adenosine triphosphate (ATP) and 1-phenylalanine were purchased from Sigma Chemical Co. St. Louis, Mo. Trisodium guanosine triphosphate (GTP), disodium creatine phosphate, creatine phosphokinase (ex skeletal rabbit muscle) and 2-mercaptoethanol were products of Koch Light Laboratories, Colnbrook, Bucks, England. Polyuridylic acid was a gift from Dr L. O. Fruholm, State Institute of Public Health, Oslo, Norway. Streptomycin sulphate was produced by Glaxo Laboratories Ltd., Greenford, England. The 13-phenylalanine C¹⁴ (uniformly labelled) was purchased from The Radiochemical Centre, Amersham, Bucks, England.

RESULTS AND DISCUSSION

It was not surprising to find that cell extracts from cells of *E. coli* carrying the Mu factor in its extrachromosomal state (11) were inhibited in the poly U stimulated synthesis of polyphenylalanine by streptomycin to the same extent as ordinary streptomycin sensitive cells (Table 1). The low level streptomycin resistance shown by the cells in this condition has already been accounted for as an impaired streptomycin uptake (10). The mechanism of the low level streptomycin resistance furnished by the Mu factor in its extrachromosomal state may be identical to the Sm resistance carried by the multiple drug resistance factor (23, 16).

In the state where the Mu factor modifies the bacterial chromosome possibly by an attachment to it (4) the cells achieve resistance to around 200 μ g streptomycin per ml when growing in complete medium. The results of the present investigation indicate that ribosomes from these cells are unchanged as compared to streptomycin sensitive ones; they are inhibited by streptomycin in their poly U stimulated synthesis of polypeptides as are ribosomes from sensitive cells. There are several conceivable ways to explain these findings.

TABLE 1

Effect of Streptomycin on Poly U-Stimulated Synthesis of Polyphenylalanine by Cell-Free Extracts from E. coli Carrying the Mu factor

Extract	Strep concentration $\times 10^{-6}$ M	L-phenylalanine inc c p m	Inhibition per cent
T71Mu	0	2787	36
	2	1809	
T71Mu str-r	0	1067	35
	2	694	
T71Mu thr his str-r	0	1090	38
	2	669	

The experimental conditions are described in "Materials and methods". The content of C¹⁴ phenylalanine per sample gave 115 000 c p m. The aliquots of S-30 fractions contained T71Mu, 21 mg, T71Mu str-r, 20 mg and T71Mu thr his str-r 17 mg bacterial protein per sample.

The ordinary streptomycin sensitive strain *E. coli* K12 T71 was inhibited to the same extent by this concentration of streptomycin (cfr 16) whereas the ordinary resistant mutant, K12 T71 str-r was completely unaffected.

Whereas both the mutator-streptomycin resistant mutants used in the present study, as well as all the ones tested, tolerate around 250 μ g streptomycin per ml, the ordinary one-step streptomycin resistant mutant used, K12 T71 str-r, has been found to grow unimpaired in the presence of 5000 μ g streptomycin per ml, and it also grows, although somewhat more sparsely, in the presence of 20 000 μ g streptomycin per ml. The S-30 fraction from this mutant is completely unaffected in the *in vitro* polyphenylalanine synthesis by 2×10^{-6} M streptomycin. This is in accordance with results from other laboratories (10, 18, 16, 12). Only in one case (22) the actual level of resistance of the streptomycin resistant strain used has been reported, and in that case the tolerance of the strain was 3-5 mg streptomycin per ml. It is, however, a generally accepted fact that the true, one-step streptomycin resistant strains have a high level resistance (12, 17). Thus, one possible explanation is that a modification of the ribosomes of the mutator-streptomycin resistant cells, increasing the level of resistance from 15 to 250 μ g streptomycin per ml results in a difference too small to be registered by the present experimental system.

It seems beyond doubt that streptomycin really interferes with the action of the ribosomes, causing the formation of aberrant protein, the evidence supporting this hypothesis is overwhelming (6, 7, 2, 14). These findings are also consistent with the genetic facts of the streptomycin system, the streptomycin sensitivity, resistance and dependence being three alleles of the same gene. This situation has been very hard to reconcile with some of the other theories on streptomycin action. There is, however, some evidence indicating that the formation of aberrant protein may not be the sole lethal action of streptomycin (21).

there may be an additional more general reaction of streptomycin with the nucleic acids (15)

Genetic evidence has been presented (8) indicating that the mutator streptomycin resistance is located at the bacterial chromosome at a site distinct from that of the ordinary streptomycin locus and apparently there is also a certain genetic distance between the two loci. The mutant *T71Mu thr his str^r* has been employed in the present study because of its former use in the genetic mapping experiments. The genetic facts agree with two possible explanations of the present results. The normal gene for streptomycin sensitivity is present in the mutator streptomycin resistant cells (8), a fact that might imply that their ribosomes are unchanged. The possibility of this resistance being due to a suppressor mechanism has formerly been suggested (8). This would result in a quantitative rather than a qualitative change which is reconcilable with the present experimental data. However, the present results do not exclude the existence of a third different mechanism of streptomycin resistance in addition to the well established ribosomal resistance and the mechanism of decreased permeability.

SUMMARY

The action *in vitro* of streptomycin on poly U stimulated polypeptide synthesis has been studied with cell free extracts from *Escherichia coli* carrying the Mu factor in the extrachromosomal as well as in the possibly attached state. The polypeptide synthesis was inhibited by streptomycin like that found in cell free extracts from streptomycin sensitive organisms suggesting another mechanism of resistance than the ribosomal resistance of the true one step streptomycin resistant mutants.

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STUDIES ON THE EPIDEMIOLOGY OF STAPHYLOCOCCAL INFECTIONS

3 *Influence of Factors Inherent in the Patient and his Operation*

By

GUNNAR LINDBOM, GUNNAR LAURELL and ÅKE GRENVIK

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In the Department of Thoracic Surgery, the epidemiology of nosocomial staphylococcal infections has been investigated since 1958. The study covers a period when—due to rapid progress in surgery and related fields—the composition of the relevant population has materially changed. Extracorporeal circulation has enabled surgical correction of many cardiac lesions, both congenital and acquired, which were formerly regarded as inamenable. This has resulted in an increase in the number of lengthy, radical and technically complicated operations on the heart. Concurrently, a decrease in surgery on patients with pulmonary tuberculosis has taken place. Moreover the indications for cardiac operations have widened. The willingness of surgeons to operate on patients who were recently regarded as too old or too debilitated has, in fact, led to a steady rise in the proportion of high-risk patients. Finally, as a result of cumulative experience, stricter standards of hygiene have been imposed, and various preventive measures against sepsis have been tested.

INTRODUCTION

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belonged to the 52 52A/80/81 complex, were characterized by the 75/77 pattern or were non-typable (Lindbom 1964). If allowance is made for changes in surveillance during the period, little evidence suggests that the total incidence of sepsis had been influenced by the increasing number of extensive operations in high-risk patients. It was nevertheless the clinical impression that the

1

1

6

published surgical experience, contamination of the wound during operation was infrequent. In 1961, approximately 2% of all patients were found to have acquired the infecting strains outside the operating theatre. About 1/3 of the septic cases were classified as autoinfection. Human carriers, healthy or infected, played a critical rôle in the intensive-care ward during the early postoperative phase, and frequently initiated a vicious circle of cross-infection.

PLAN OF THE PRESENT STUDY

Although calculation of the over all rate of postoperative infections reflects the total picture of sepsis it fails to identify particularly infection prone groups of patients. The method of surveillance therefore included relevant clinical and bacteriological data, to permit calculations of specific infection attack rates. It was drawn from three sources: wound healing cards, laboratory notes, and case records.

Most patients passed through several hospital environments in the unit: i.e. the nursing ward, the operating theatre, and the intensive care ward, where they were exposed to a variety of conditions for varying periods. Previous studies had emphasized the importance of the early postoperative phase in the intensive care ward. Hence the interaction was to be studied between postoperative patients and the environmental pool of staphylococci as well as the dependence of the environmental flora on the presence of staphylococcal disseminators.

In view of the high rate of autoinfection in 1961, special attention was also focused on the nasal carrier state of the patients, and nasal chemotherapy was instituted.

The results and conclusions of the study will be presented in three reports dealing with different aspects: (a) the influence of factors inherent in the patient and his operation on the rate of postoperative sepsis; (b) the influence of nasal chemotherapy on the carrier state in patients; and (c) the influence of environmental factors during the early postoperative phase.

MATERIAL AND METHODS

The lay-out of the wards and the basic bacteriological technique were the same as those previously described (Lindbom 1964) but the epidemiological survey was more comprehensive. It included regular carrier examinations, standardized environmental examinations in the intensive care ward and a pilot study of the operating theatre.

Background

General Observations. The study took place from January 1st to December 31st 1962 inclusive. A total of 536 patients were admitted to the unit. There were 313 operations. Their duration ranged from 1.2-9.6 hours (average 3.3). Due to the high incidence of lengthy procedures on the open heart, the average number of operations per operating day usually ranged from 1.1-1.3. In May, June, September and December it rose to 1.6-1.7 because of an increased number of relatively short operations.

The "average" patient remained in the unit for 29.5 days, of this time he spent 9.5 days in the ward undergoing a preoperative work up, 4.5 days postoperatively in the intensive-care ward and, finally, another 15.5 days in the ward before being transferred to another unit or another hospital. Table 1 shows the average duration of these three phases for certain cardiac and pulmonary operations.

Respirator Treatment. A total of 80 patients were tracheotomized. The duration of treatment ranged from 1-51 days, in 2/3 of the patients it lasted for 3-8 days (average 7.8).

Antimicrobial Policy. Prophylactic antibiotics were given routinely to about 50 per cent of the patients preoperatively and to over 90 per cent postoperatively. Penicillin G and chloramphenicol were the drugs of choice and were frequently combined. Penicillin was given to 20 per cent of the patients preoperatively and to 32 per cent postoperatively. Chloramphenicol was given to 10 per cent and 41 per cent respectively. Other antibiotics were generally not used. The importance of chloramphenicol in the intensive care ward had been replaced by a Starr-Edwards prosthesis, the patient received penicillin and chloramphenicol for a week after operation via a catheter in the superior vena cava.

Nasal Disinfection. Chlorhexidine neomycin was used. **Collection of Data.** Sources: i.e. wound infections isolated in the unit, dependent staphylococci, of the

case records of all operated patients. They were to serve as a control of the efficiency of the other two sources, and to permit calculations of specific infection attack rates.

Selection of Patients. All patients whose postoperative course was complicated by infection were included. The epidemiological study dealt only with patients who acquired staphylococcal infections.

TABLE 1

Average Duration of Phases of Hospitalization in Some Common Cardiac and Pulmonary Operations

Surgical procedure	Phases of hospitalization		
	Preoperative days	Operative hrs	Postoperative days
Total valve prosthesis	12	5.2	20 (11)
Transventr. dilatation	10	2.3	17 (4)
Closure of ASD	5	2.6	12 (4)
Closure of VSD	10	3.9	23 (5)
Beck's operation	25	1.7	23 (4)
Resection of aortic coarct	4	2.6	6 (3)
Division of patent ductus	0	2.1	6 (3)
Pulmonectomy	15	3.5	20 (6)
Lobectomy	14	3.3	12 (3)
Segmental resection	13	2.9	13 (5)
Expl. thoracotomy	10	2.2	14 (4)

Bracketed figures denote duration of postoperative intensive care

The final decision whether a patient should be classified as infected was made by a thoracic surgeon (G. K.), who saw most patients and participated in their operations. In general, the classification presented no problem. The absence of a bacteriological verification would however have excluded several suspect cases of septicæmia where clinical data and/or microscopical diagnosis were accepted as presumptive evidence of septicæmia.

Definitions. A postoperative wound infection was defined as a complication in which bacteria were not only present in the incision, but also elicited a local or systemic response.

When leucocytosis—was present in a patient more than 5 days after operation he was classified as a presumptive case. In patients receiving antimicrobials directly via vena cava catheters bacteriological verification was frequently impossible. The aetiological diagnosis was inferred from the presence of staphylococci elsewhere in concomitant septic lesions such as empyema, tracheal secretion, and a septic wound.

Partial atelectasis was frequent postoperatively. Varying degrees of pulmonary opacification on chest X rays and "infected" sputum were not accepted as reliable criteria of bronchopneumonia. Consequently only cases where the diagnosis was established at autopsy are included.

The term *staphylococcal diarrhoea* denotes a postoperative condition characterized by moderate to profuse stools from which enterotoxin B producing staphylococci were isolated.

Staphylococcal disease was classified as *minor* when due to a trivial wound infection whose main importance was epidemiological, i.e., as a potential source of pathogens. *Major* infections were associated with septicæmia, empyema, sternal osteitis, staphylococcal diarrhoea or wounds with invasion and destruction of tissue.

Bacteriological Routine

Carrier Examination. (a) *Patients.*—Nasal and throat swabs were taken from patients on the day of admission or as soon after as possible. Patients were also

PLAN OF THE PRESENT STUDY

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In open heart surgery when a diseased cardiac valve had been replaced by a Starr-Edwards prosthesis, the patient received penicillin and chloramphenicol for a week after operation via a catheter in the superior vena cava.

Nasal Disinfection. All patients underwent nasal chemotherapy using a chlorhexidine-neomycin cream and/or a framvetin-gramicidin nasal spray.

Collection of Data. In 1961 information was collected from two independent sources - wound healing cards and continuous registration of all staphylococci isolated in the unit. In the present study a third source was added - a review of the

diagnosis in 3 cases where post-mortem findings supported the diagnosis of septicæmia. The diagnosis of "bronchopneumonia" in 11 cases was based solely on the post-mortem examination, bacteriological verification was possible in 3 cases, whereas no specimens were obtained from the remaining 8.

TABLE 2
Total Spectrum of Infectious Complications in 58 Patients

Type of complication	Clinical diagnosis		Total no
	Verified	Not verified	
Wound	39	5	44 (18)
Wound abscess	3	3	6 (3)
Retrosternal abscess	2	1	3
Osteitis	1	1	2
Bronchopneumonia	3	3	6 (2)
Empyema	4	—	4 (3)
Septicæmia	7	10	17 (6)
Staph. diarrhoea	6	—	6 (2)
Total	60	23	83 (34)

Bracketed figures denote number of single lesions

TABLE 3
Postoperative Infections Grouped According to Site of Operation

Site of operation	Infections		No of infections
	Major	Minor	
Heart and great vessels	23 (15)	17	40
Lungs	5 (2)	4	9
Miscellaneous	7 (2)	2	9
Total no of infections	35 (19)	23 (1)	58

Bracketed figures denotes number of deaths

Importance of Postoperative Infection: Septic complications varied from minor episodes in 23 patients to major illness in 35 patients, which contributed to the death of 19. Two-thirds of all infections were classified as "major" (Table 3).

In 23 patients who were seriously ill with staphylococcal disease, the course was progressive, with a tendency to necrosis, abscess formation and septicæmia. It could be arrested in 7 patients by intense antimicrobial therapy or additional operations but progressed rapidly to death in the remainder. Tissue destruction subsequent to sepsis nullified the expected result of surgery in 6 patients, and contributed to the death of 5.

Only a minority of patients remained in the unit for more than a

swabbed on the day of operation and daily during their stay in the intensive-care ward (b) Personnel—Nasopharyngeal swabs were obtained from regular members of the personnel every other month.

Septic Complications. As soon as clinical symptoms and signs suggestive of infection appeared, specimens were collected for culture, to verify the diagnosis and to determine the aetiological agent.

Bacteriological Technique. The bacteriological technique was described in earlier reports (Jaurell & Lindbom 1961). The term "staphylococci" as used in this report refers only to coagulase positive strains. All staphylococci were phage typed with the basic set of 21 international phages in addition to phage λ SG (Wallmar 1954). The technique was that described by Blair & Williams (1961). The disc diffusion method of Friszon *et al.* (1954) was used for sensitivity determinations.

Formation of enterotoxin II was determined by a method described by Hallander (1965). Staphylococci to be examined were streaked onto solid media covered with cellophane, and incubated for 24 hours. The supernatants obtained after centrifugation were tested for the presence of enterotoxin by the gel diffusion technique.

Bacteriological Data. Information in this study derived from approximately 8000 cultures including 1179 clinical specimens and 6716 specimens collected to map the epidemiological background.

Part 1 of the study, which deals with the patient and his operation is based on 210 strains of staphylococci isolated from clinical specimens.

RESULTS

1 Infectious Complications

Incidence of Infection. Postoperative infection developed in total in 58 among 313 operated patients. *Staph aureus* was isolated from 41. The over all infection rate was 18.5 per cent, the staphylococcal infection rate 13.1 per cent. The total number of septic lesions in these 58 patients was 88. Single lesions occurred in 34 patients and multiple in 24.

Efficacy of Surveillance Methods. Only by a thorough review of case records could 58 cases of postoperative septic complications be disclosed. If this is accepted as the true number of infections, the accuracy of "wound-healing cards" was just over 65 per cent (38 cases), and that of bacteriological records about 74 per cent (43 cases). As information from these two sources did not completely overlap, they detected just under 80 per cent (46 cases) of all infections. The inadequacy of the reporting system was due either to the absence of cards concerning emergency operations, operations on children and those not strictly on the heart and lungs, or to inadequate or missing cultures from suspected septic lesions.

Reliability of Classification. The extent to which a clinical diagnosis could be confirmed bacteriologically is shown in Table 2. Verification was possible in about 74 per cent of all lesions classified as septic. Whereas the diagnosis was confirmed in 89 per cent of all septic wounds, that of septicaemia had to be based on clinical data in 10 of 17 cases. All unconfirmed cases had received massive doses of antibiotics (including methicillin) when blood samples were collected. The outcome was fatal in 6 verified and 8 unverified cases. Bacteriological cultures from relevant autopsy tissues failed to confirm the clinical

diagnosis in 3 cases where post-mortem findings supported the diagnosis of septicaemia. The diagnosis of "bronchopneumonia" in 6 cases was based solely on the post mortem examination, bacteriological verification was possible in 3 cases whereas no specimens were obtained from the remaining 3.

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In 23 patients who were seriously ill with staphylococcal disease, the course was progressive with a tendency to necrosis, abscess formation and septicaemia. It could be arrested in 7 patients by intense anti-microbial therapy or additional operations but progressed rapidly to death in the remainder. Tissue destruction subsequent to sepsis nullified the expected result of surgery in 6 patients, and contributed to the death of 5.

Only a minority of patients remained in the unit for more than a

few weeks after operation, although several with serious infections had to be hospitalized for periods ranging from 2-4 months

Staphylococcal Diarrhoea Of particular interest were 6 patients with postoperative diarrhoea. In 4, it was part of a clinical picture that included a septic wound combined with bronchopneumonia or septicaemia. All patients had positive cultures of *Staph aureus* in specimens of their stools, and concomitant septic lesions. The strains isolated were identified by phage typing as 75/77 or Thorax I (cf. the following). Both were found to produce enterotoxin B. An interesting feature was the frequent association of diarrhoea with tracheobronchitis and/or bronchopneumonia. Thus, severe staphylococcal disease characterized by simultaneous infection of the gastro-intestinal and respiratory tract occurred in 4 patients, 3 of whom later succumbed to their infection. It was typical of this disease pattern that patients harboured and liberated large numbers of staphylococci and caused massive environmental contamination. Further details of the epidemiological importance of these disseminators will be published separately (Lundbom & Laurell).

It is noteworthy that although enterotoxic strains produced septic complications in a total of 16 patients (including 2 double infections), there were only 2 cases of isolated staphylococcal diarrhoea. In the others, the clinical picture was a combination of infection of mucous membranes, usually associated with septicaemia and other severe forms of disease.

Association Between Type of Operation and Rate of Sepsis

Heart and Great Vessels In total 175 operations were performed. Certain operative characteristics were used to divide the material into two main groups: open and closed heart operations (Table 4). These were further divided into two subgroups, i.e. acquired and congenital defects, in which operations were listed according to the underlying disease.

Whereas operative correction of congenital cardiac deformities is fairly well standardized, it is in a developmental stage for acquired lesions. For technical information on cardiac operations reference is made to Björk (1964). It should be noted that valvular replacement was performed only in advanced cases. Respirator treatment was used in patients with an enlarged heart and pulmonary hypertension to lessen respiratory work and diminish the cardiac output. Seventy patients were tracheotomized when prolonged treatment was anticipated.

The term open heart operation covers various intracardiac procedures performed on 95 patients. Extracorporeal circulation (ECC) was used in 76 and hypothermia in 26. In 7 hypothermic patients ECC was subsequently found to be necessary. Altogether 43 patients had acquired mitral and/or aortic valvular deformities. Prosthetic replacement was performed in 41 patients: mitral annuloplasty in 1 and aortic commissurotomy in 1. A Starr-Edwards ball valve prosthesis was used in 38 operations, including 2 re-operations to replace 32 aortic and 10 mitral valves. A double aortic and mitral prosthesis was used in 2 patients. A Muller prosthesis was used in 2 patients and a Hufnagel prosthesis in 1. Hypothermia was used

in 20 patients with an atrial septal defect (with or without anomalous pulmonary return) 3 with pulmonary stenosis and 3 with coarctation of the aorta

TABLE 4
Operations on the Heart and Great Vessels

Operative procedure	Structural changes	Operated cases	No of Infected cases	Infections %
Open heart operation	<i>Acquired</i>			48.9
	AI \pm AS	29	14	
	MI \pm MS	10	3	
	AI \pm VI	4	4	
	Ventr aneurysm	3	1	
	Misc	1	1	
	<i>Congenital</i>			22.9
	Tetralogy of Fallot	4	1	
	VSD	13	3	
	ASD (see)	23 (20)	5 (4)	
	Misc	8 (3)	2	
Closed operation	<i>Acquired</i>			6.6
	MS	12	8	
	Angina pectoris	11	2	
	Adams Stokes syndrome	29	1	
	Misc	9	1	
	<i>Congenital</i>			10.5
	Coarctation of aorta	8	2	
	Patent ductus art	11	0	
Total		175	40	22.9

A aortic
M mitral
I insufficiency
S stenosis
ASD atrial septal defect
VSD ventricular septal defect

Bracketed figures denote number of operations under hypothermia

The over all rate of sepsis in this group of 175 patients was 22.9 per cent. It was 35.8 per cent in patients undergoing open heart surgery, 48.9 per cent in patients with acquired valvular deformities as compared to 22.9 per cent in those with various congenital anomalies. Postoperative infection occurred in 21 patients (53.5 per cent) with a ball valve prosthesis in 17 cases after replacement of aortic valves and in 5 after mitral replacement. Sepsis developed in only 4 of 26 hypothermic operations (15.4 per cent).

It is noteworthy that 27 of 70 thorotomized patients (38.6 per cent) became infected.

Closed cardiac operations carried an infection rate of 7.5 per cent. Four patients with acquired lesions and 2 with congenital deformities

became infected. Two patients out of 11 undergoing Beck's operation developed sepsis. Wound sepsis occurred in one patient, and staphylococcal diarrhoea in the other, whose parietal wound was perfectly healed. Resection of aortic coarctation in 8 patients was followed by trivial sepsis in 2. In contrast, 11 patients with a patent ductus and 12 with mitral stenosis remained free from postoperative sepsis.

Operations associated with a high rate of sepsis were usually of long duration. On an average, open correction of acquired valvular deformities, ventricular (VSD) and atrial septal defect (ASD) lasted for 5.2, 3.9 and 2.6 hours, respectively. There was, however, no direct evidence of a time-risk relation, since ASD and VSD operations were associated with a similar rate of sepsis, despite a marked difference in duration, whereas transventricular dilatation of stenotic mitral valves (averaging 2.3 hours) remained entirely free from septic complications. Furthermore, the use of extracorporeal circulation did not in itself predispose to sepsis, as congenital defects made accessible to surgery by hypothermia (ASD) and ECC (VSD) were accompanied by similar attack rates, 20 per cent and 23 per cent, respectively.

A closer correlation seemed to exist between the rate of sepsis and patient factors. Thus, various host characteristics, such as duration of underlying disease and cardiac reserve, showed a positive correlation to the incidence of sepsis. Valvular replacement by a prosthesis was performed only in advanced cases of enlarged heart (700-900 ml/m, BSA) in patients who frequently had pulmonary hypertension and some degree of myocardial failure. Generally, they were in need of prophylactic respirator treatment and were tracheotomized in the theatre.

Clinically, infections were trivial in 5 patients and severe in 16 after prosthetic replacement of diseased valves. They contributed to the death of 13 patients. Generally, they were due to a combination of a septic wound and/or a deep abscess with septicæmia. Most clinical specimens yielded a polybacterial flora of *Staph aureus*, *Staph albus* and/or enteric gram-negative bacteria. The last-mentioned were isolated only from patients undergoing therapeutic methicillin treatment. In contrast, only 2 septic complications subsequent to correction of congenital cardiac anomalies were classified as serious. Sternal osteitis developed in one patient, and a retrosternal abscess in the other. Cultures yielded *Staph aureus* alone or in combination with *Staph albus*.

More than 45 per cent of the septic complications following prosthetic replacement of deformed valves were due to autoinfection.

Closed operations were associated with 5 minor wound infections and 1 case of isolated staphylococcal enterocolitis.

Lungs. Data on 72 pulmonary operations are summarized in Table 5. The main indications for surgery were malignant tumours (25 cases), tuberculosis (20 cases), and bronchiectasis (6 cases). Pneumonectomy

TABLE 5
Pulmonary Operations

Pulmonary Operations												
Clinical diagnosis	Operative procedure										Infections %	
	Pulmonectomy ± Thoracoplasty		Lobectomy ± Segment resection		Thoracoplasty		Segmental resection		Other operations incl thoracotomy			
	Operated cases	Infected cases	Operated cases	Infected cases	Operated cases	Infected cases	Operated cases	Infected cases	Operated cases	Infected cases		
Tuberculosis of lung	2	0	14	1	3	0	1	0	1	0	50	
Tumour of lung	20	8	4	0	0	0	8	0	1	1	219	
Bronchiectasis	0	0	6	1	0	0	0	0	0	0	167	
Other pulm processes	0	0	0	0	2	0	12	0	12	0	0	
Infections %	27.3		8.3			0			4.8		12.5	

was performed in 21 cases and lobectomy in 22. About 90 per cent of the former were performed on patients with malignant tumours and 60 per cent of the latter on patients with tuberculosis.

Both pneumonectomy and lobectomy involved transection of previously infected tissues. Atypical infections distal to carcinous bronchial obstruction were common prior to surgery in pneumonectomized patients. The average age was also higher (46.2 years) than that in lobectomized patients (30.7 years). As respiratory difficulties were anticipated in 7 patients they were tracheotomized to permit the use of a mechanical ventilator. Intercostal drainage tubes were placed in the thorax to maintain pulmonary expansion.

The total number of postoperative infections was 9 giving a total sepsis rate of 12.5 per cent. Postsurgical infections complicated 27.3 per cent of all pneumonectomies and 8.3 per cent lobectomies.

Infection attack rates for pneumonectomy and lobectomy were not directly related to the length of operation (3.5 and 3.3 hours respectively). Again factors inherent in the patient—such as age, underlying disease, previous infection of the lung, nature and extent of ventilatory disturbances—tended to obscure the influence of increasing duration of operation.

After pneumonectomy infectious complications appeared in 6 patients with malignant tumours. They were serious in 5 and a contributory cause of death in 2. All patients had been tracheotomized. The clinical picture was characterized by septic wounds plus tracheobronchitis, bronchopneumonia and in some cases empyema. Clinical specimens yielded both *Staph aureus* and gram negative enteric bacteria. A trivial wound in the 6th patient—who had not been tracheotomized—was due to *L. coli* and *Staph albus*.

Minor septic wound from which *Staph albus* and gram negative bacteria were isolated developed in 2 lobectomized patients.

Miscellaneous. In this heterogeneous group of patients 9 out of 66 operations were complicated by postoperative infection. 5 were associated with vascular operation, 2 with tracheal and 1 with gastrointestinal. The total incidence of sepsis was 13.6 per cent.

The clinical picture was usually characterized by a septic wound or an abscess. There was only 1 case of septicæmia. Surgery on the trachea and trachea was complicated by 5 serious infections, 2 of which ended fatally. *Staph aureus* was the dominant organism. Two thirds of all infections were monobacterial, $\frac{1}{3}$ due to mixtures of *Staph aureus*, *Staph albus* and less commonly to enteric gram negative organisms.

Tracheostomy. Septic complications developed in 34 out of 80 tracheotomized patients (42.5 per cent). 23 were caused by *Staph aureus*.

2 Bacteriological Findings

The results of bacteriological examination of clinical specimens are summarized in Table II. Pure cultures were found in 33 patients with postoperative infections and mixed cultures in 25. *Staph aureus* was the predominating organism. It was isolated from 70.7 per cent of all infections while *Staph albus* was found in 36.3 per cent and various enteric gram negative bacteria such as *E coli*, *Proteus* and *Pseudomonas* in 25.9 per cent. *Staph aureus* was thus about twice as frequent as *Staph albus* and three times as frequent as gram negative bacteria. Their relative aetiological importance i.e. their ability to initiate infection under existing conditions was estimated in 33 patients with monobacterial sepsis. *Staph aureus* had produced 75.0 per cent of these infections causing more than 3 times as many infections as *Staph albus* and 25 times as many as enteric gram negative bacteria.

TABLE 6

*Bacteria Isolated in Postoperative Infectious Complications in 55 Patients**

Organisms	No. of infectious complications
<i>Staph aureus</i>	41 (95)
<i>Staph albus</i>	21 (7)
Gram negative bacteria	15 (1)
None	1
	78 (33)

*No material for bacteriological examination was obtained from 3 patients.

Bracketed figures denote number of pure cultures.

Phage Typing Results. By means of phage typing 81 different lytic patterns could be distinguished by conventional principles of strain differentiation. About 60 per cent of all staphylococci had to be re-typed at RTD $\times 1000$ resulting in a great number of somewhat broad lytic patterns that were often difficult to interpret. The main problem was presented by two strains characterized by wide pattern involving phages of groups I and III. One has been denoted as Thorax "BP" (basic pattern). Its patterns (including moderate reactions) were basically alike RTD $\times 1000$ 52 24 80 45 67 42 75 34/75 77 83A but did not show complete correspondence. Deviations from this basic pattern involving the gain or loss of strong reactions occurred frequently in a set of cultures obtained at intervals from the same patient. Strains with this pattern have been regarded as related without further epidemiological evidence. Distinct from the "BP" staphylococcus was a strain Thorax "I" (inhibition) which was characterized by reactions of inhibition produced by phages 81 45 67 47 83A and less commonly 52 52A 7 42F and 75. Although deviations from the

pattern of inhibition reactions were common, these strains showed fairly good reproducibility

Among 81 recognizable staphylococcal strains, 24 were recovered from various septic lesions in 41 patients. Five distinct strains produced more than 2 infections each. They have been denoted as "epidemic." Such strains, recovered from 15 septic cases, were identified by well-defined lytic patterns, *i.e.* the 52/52A/80/81 complex (4 cases, 9.8 per cent), 75/77 (8 cases, 19.5 per cent) and RTD $\times 1000$: 53/42E (3 cases, 7.3 per cent). Staphylococci characterized by wide complex patterns were recovered from 18 patients, Thorax BP (8 cases, 19.5 per cent) and Thorax I (10 cases, 21.4 per cent). As cultures from 3 patients with 75/77 infections also yielded either Thorax BP or Thorax I strains, these 5 epidemic strains together accounted for 31 infections, *i.e.* 75.6 per cent of all staphylococcal infections. The remaining 10 cases were all sporadic infections.

Antibiograms Approximately $\frac{2}{3}$ of all staphylococci isolated from clinical specimens were resistant to both penicillin and tetracycline. They have been classified as "hospital" strains. Resistance to chloramphenicol was present in just over 50 per cent. Resistance to methicillin was not observed. As most strains were represented by rather small materials, resistance patterns were determined only for staphylococci which were commonly found in clinical specimens. Antibiograms for 75/77, Thorax BP and Thorax I strains are given in Table 7.

TABLE 7
Relative Percentage of Resistance to Antibiotics in 3 Common Disease Producing Strains

Phage pattern	Resistant strains %				
	Pe	Fr	Te	Ch	Mc
75/77	95	62	55	68	0
Thorax BP	78	75	92	72	0
Thorax I	72	63	81	33	0

75/77 This homogeneous strain, believed on epidemiological grounds to have a common origin, showed striking individual variations in resistance to erythromycin and tetracycline. There were 2 "variants" one was present almost continuously in the unit up to summer closure; the other was brought there in the autumn by carriers from other parts of the hospital. The former was highly resistant to all antibiotics except methicillin, whereas the latter was usually sensitive to erythromycin and tetracycline, sometimes also to chloramphenicol.

Thorax BP This strain gradually grew more resistant, or sensitive isolates became infrequent, the great majority were of intermediary resistance.

Thorax I When introduced into the unit this strain was resistant to penicillin and tetracycline, and sensitive to erythromycin and chloramphenicol. Within a month erythromycin resistant isolates were common.

3 Source of *Staphylococci*

Extensive studies were undertaken to determine when and where pathogenic staphylococci were acquired. As a detailed account will be published separately, the results shall be summarized briefly. Of 41 infected patients, 14 (34.2 per cent) were carriers of the infecting strain already at the time of operation. Contamination of tissues may have taken place either in the theatre or in the intensive-care ward. They have been classified as *endogenous* (autoinfections). Infections that were regarded as *exogenous* theatre acquired developed in 4 patients (9.8 per cent). Among the remainder 22 (53.7 per cent) were acquired in the intensive care ward and 1 (2.4 per cent) in the nursing wards. They have been classified as *exogenous, ward acquired*.

In 23 tracheotomized patients 10 infections (43.5 per cent) were classified as endogenous, 1 (4.4 per cent) as exogenous theatre acquired and 12 (52.2 per cent) as exogenous ward acquired.

In 32 septic cases (78.1 per cent) human beings were established as the ultimate source of disease producing staphylococci.

DISCUSSION

In 1962 the over all infection rate was 18.5 per cent and the staphylococcal infection rate 13.1 per cent. These rates were higher than in 1961 when they comprised 13.9 and 10.6 per cent. But the rise was in all probability an artefact due to closer surveillance in 1962. The accuracy of wound healing cards as the single source of information was shown to be no better than 60 per cent and that of bacteriological records 74 per cent. As there was not complete overlapping of information supplied by these two sources their combined use detected about 80 per cent of all infections. A time consuming review of the records of all operated patients added another 12 unreported infections. Obviously 1 of every 3 infections would have remained unknown had not the control been included in the method of surveillance.

Apart from the review of case records the criteria of infection had shifted. In earlier reports there had been the bias of purely bacteriological criteria whereas the final decision whether or not a patient was to be classified as infected was now made by a surgeon. Bacteriological verification was possible in about 74 per cent of all septic lesions. Unfortunately the clinical suspicion of septicaemia was hard to verify. Routine procedures fail to confirm the clinical diagnosis in 10 of 17 patients, all of whom were receiving massive doses of antibiotics frequently through a catheter in the superior vena cava. It is

recognized that these unconfirmed cases were not necessarily septicemias but could have been due to other causes including toxic reactions to antibiotics and the postpericardiotomy syndrome.

The clinical importance of postoperative staphylococcal disease is obvious from the high proportion of serious infections. 73 were classified as severe. 19 died. Although infection contributed to the fatal outcome in many cases its importance could not be determined precisely since both infection and subsequent death occurred primarily in patients with serious underlying disease. The immediate cause of death was most often failure of the cardiorespiratory system. Most significant was that the number of serious staphylococcal infections had increased from 14 in 1961 to 35 in 1962, i.e., a rise of 150 per cent whereas the mortality in such cases remained at about 50 per cent.

Taken as a group operations on the heart and great vessels carried a higher risk of postoperative infections than pulmonary operations, the incidence of septic complications being 22.9 per cent in the former as compared to 12.5 per cent in the latter. Detailed analysis of the patients, their underlying illness and type of operation revealed, however, that this difference was not due to any specific vulnerability of the heart to infections.

By grouping cardiac patients according to the nature of the underlying disease and the type of surgical procedure it became evident that the infection attack rate ranged from 0 to 53.5 per cent. Whereas mitral valvulotomy was never complicated by infection, sepsis developed in every second patient with a Starr prosthesis. Similarly the rate ranged from 0 per cent in segmental resection to 27.3 per cent in pneumonectomy. Obviously the risk of sepsis was related to factors inherent in the patient and to his operation.

Clinically the nature of the infection also varied from group to group. Patients undergoing prosthetic replacement of diseased heart valves were particularly prone to progressive infections such as tracheobronchitis and/or bronchopneumonia, a deep abscess and septicemia whereas other cardiac patients were subject to circumscribed lesions. A similar difference characterized pneumonectomized and lobectomized patients. Tracheobronchitis and/or bronchopneumonia, empyema and a septic wound developed in the former in contrast to the latter in whom trivial septic wounds appeared.

A comparison of sepsis rates for open and closed cardiac operations, 38.5 and 7.5 per cent respectively, showed that the difference was significant ($p = 0.05$). But this difference was influenced by numerous factors other than the type of surgical procedure which was used to define the two groups. Thus the risk of sepsis was significantly higher in patients with enlarged hearts, reduced cardiac reserve and frequently also secondary pulmonary changes. They underwent prolonged intracardiac operations made possible by extracorporeal circulation which is known sometimes to cause harmful side effects. Their inci-

sions were wide, foreign bodies were implanted

Viable staphylococci in numbers sufficient to cause infection reached a susceptible site in even Starr prosthesis, but not in patients undergoing Therefore, the risk of staphylococcal sepsis in this pattern, was influenced not only by the presence of organisms to invade the tissues, but by the sum of a large number of factors. This concept is supported by Lidwell's statistical analysis of the P.H.L.S. investigation, where he demonstrated that the procedures in themselves carry a low basic risk. When associated with certain adverse factors, however, the risk is significantly increased multiplicatively when they coexisted.

The small somewhat heterogeneous material does not permit evaluation of the association between the rate of

(Lidwell 1961, Barnes 1962) An age risk was observed. It was evident in patients on whom pulmonary resection was performed. Pulmonectomized patients were, on average, older than lobectomized patients. More significantly, perhaps, that the case histories of the former group showed a high incidence of "atypical" pulmonary infection prior to surgery. In fact, many of these patients had been tracheotomized because they had anticipated postoperatively. If age was a factor, it was lost among the effects of many other factors leading to the septic outcome.

Operations associated with high rates of mortality, lasting for more than 25 hours. About one third of the patients by infection had lasted for 25-35 hours, and about one half for more than 35 hours. There was an accumulation of lung abscesses in the range and of open heart operations in the field of comparable operations, infected and noninfected. A direct time risk relation. Although of similar duration, lobectomy (33 hours) and lobectomy (35 hours) showed strikingly different infection attack rates (27% vs. 100% respectively). Still longer operations such as aortic arch replacement (duration 52 hours), carried extremely high mortality. Surgery became more extensive, host factors and operative status—seemed to become increasingly important. Sepsis. Again, tracheostomy was a common

factor. As only 10% of patients died of RTD by the time of surgery, the highly complex nature of the disease, characterized by wide variation in BP, was not fully appreciated until 1953, 54, 75, 77, and 78. The incidence of inhibition of growth was 7, 42E and 75% respectively. It is currently hard to interpret only by

the presence of organisms in the blood of patients having received chloramphenicol antibiotic or respiratory staphylococcal chemo

therapy. Special attention to diarrhoea. They were caused by a variety of organisms. The sole cause of the disease is the inability to control the infection. The infection posed a serious problem for patients and their families.

The patient's condition was improving. It was a difficult and a step towards a successful outcome. The structure of the disease was a turnover of the disease. The characteristics of the disease were biological.

The patients were in a state of

infected patients. The time-risk relation, if present, was also obscured by other factors.

Postoperative septic complications were observed in a total of 34 among 80 tracheotomized patients. Tracheostomy was a common denominator in 58.6 per cent of all infections. Its epidemiological rôle could be established by the high incidence of patients who were colonized by prevalent staphylococcal strains. By-passing the protective function of the nasopharynx, staphylococci from the air, adjacent sites of wound infection or contaminated suction catheters colonized traumatized mucous membranes. Particularly infection-prone were tracheotomized patients with a median sternotomy, in whom slow-healing single-wound cavities frequently appeared.

As a rule, the staphylococcus seemed to need an anatomical lesion to by-pass normal defense barriers. Remaining *in situ* for 3-7 days post-operatively in most open-heart operations, a cannula, a vena cava catheter and several drainage tubes presented ambient staphylococci with 4-5 pathways by which they could gain access to intrathoracic structures in close proximity to foreign bodies, such as a valve prosthesis and an aortic or myocardial graft.

Open pathways may also help to explain why approximately 30 per cent of all infections were due to ward-acquired, exogenous staphylococci, and why endogenous staphylococci were responsible for close to 50 per cent of infections in those carriers who underwent open-heart surgery. There were, in fact, outbreaks of autoinfection among infection-prone patients during periods when exposure to epidemic strains was infrequent or of short duration (Lindbom & Laurell). Whatever the mechanism, certain operations seemed to induce in the patient a state of increased susceptibility to his own staphylococci. This is all more noteworthy as there was only a single primary infection - a case of *Klebsiella bronchopneumonia* - that could be attributed to endogenous gram-negative bacteria.

Staph aureus was the predominating organism, responsible for more than 3 times as many monobacterial infections as *Staph albus* and 25 times as many as gram-negative bacteria. Although of secondary importance, *Staph albus* caused serious monobacterial infections in 3 patients who had a diseased valve replaced by a prosthesis. Compared to *Staph aureus*, other organisms seemed to have less ability actually to initiate sepsis, even in infection-prone patients. As far as gram-negative bacteria are concerned their rôle was primarily that of a secondary invader in cardiac patients receiving methicillin and in certain tracheotomized lung patients, or of a contaminant in some trivial wound infections.

Phage typing revealed that 5 strains of *Staph aureus* produced 75.6 per cent of all infections. In contrast to 1961, staphylococci belonging to the 52/52A/80/81 complex played a minor rôle, whereas staphylococci identified by a clear cut 75/77 pattern remained entrenched in the

unit, or were repeatedly reintroduced from other reservoirs. As only about 40 per cent of all staphylococci were typable at RTD by the basic international set of phages patterns were often highly complex. Particularly hard to interpret were two strains, characterized by wide patterns involving phages of groups I and III. One, Thorax BP, was lysed at RTD $\times 1000$ by phages 52, 52A, 80, KS6 7, 42E, 53, 54 75 77 and 83A. The other, Thorax I had a fairly stable pattern of inhibition with phages 81, KS6 47, 83 and, less commonly, 52, 52A, 7, 42E and 75. When present simultaneously in the unit, they were frequently hard to differentiate. Initially, this could be done with certainty only by titration.

Antibiotics were important determinants of the types of organisms present in the unit. Approximately 60 per cent of the patients harboured staphylococci resistant to penicillin, tetracycline and chloramphenicol. When patients received large doses of a single antibiotic or multiple drugs in combination, they acquired resistant hospital staphylococci rapidly and with amazing frequency, despite nasal chemotherapy.

Two tetracycline resistant staphylococcal strains merit special attention. Isolated originally from patients with staphylococcal diarrhoea they were shown to produce enterotoxin B (Hallander 1965). They were identified by the 75/77 and Thorax I patterns. Clinically, they caused a multiform disease that included one or more of the following symptoms: a septic wound, bronchopneumonia, diarrhoea and septicaemia. Diarrhoea occurred in $\frac{1}{2}$ of the patients, only rarely was it the sole sign of infection. These strains seemed to have a special affinity to mucous membranes and the ability to induce profuse secretion of sputa or stools resulting in massive environmental contamination. There was no evidence that infection prone patients were predisposed to complications by such strains, although tracheotomized patients played a central rôle in a vicious epidemic circle either as victims or disseminators or both.

In roughly $\frac{3}{4}$ of all infections, a person—most commonly a patient—could be established as the ultimate source of disease producing staphylococci. It was evident that factors inherent in the patient and his operation were important determinants of whether a given staphylococcus in a given patient was to cause disease. It was nevertheless equally obvious that numerous external factors—such as structure of the unit, intramural movements of the patients and rate of turnover contributed to the creation of a reservoir where characteristics and prevalence of staphylococci influenced the epidemiological pattern.

SUMMARY

Postoperative septic complications developed in a total of 28 patients (18.5 per cent). *Staph aureus* was the predominating organism at

counting for 41 infections. About $\frac{1}{3}$ were due to endogenous staphylococci frequently acquired in hospital but before admission to the thoracic surgery unit. Thirty-five infections were serious, contributing to the fatal outcome in 19 cases. Enterotoxin B producing staphylococci played an important epidemiological rôle.

Intrathoracic operations were associated with wide variations in the specific infection attack rate, which ranged from 0 per cent after mitral valvulotomy to 53.5 per cent after prosthetic replacement of diseased heart valves. The incidence of postoperative sepsis showed a positive correlation to numerous non-bacterial factors, such as underlying disease, functional state of heart and lungs, type of surgical procedure, postoperative tracheostomy, indwelling catheters in the superior vena cava and multiple drainage tubes. These factors were primary determinants of whether a given staphylococcus in a given patient was to cause disease. They also explain periodic outbreaks of non-epidemic infections due to endogenous staphylococci. Numerous other factors determined which types of staphylococci patients were exposed to.

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STUDIES ON THE EPIDEMIOLOGY OF STAPHYLOCOCCAL INFECTIONS

4 Effect of Nasal Chemotherapy on Carrier State in Patients and on Postoperative Sepsis

By

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Received 22 vii 66

Recent studies of hospital infections caused by *Staph aureus* have emphasized that carriers play a central rôle in the epidemiological cycle, either as victims, or sources, or both. The association between nasal carriage and an increased risk of postoperative sepsis was first described by Williams *et al* (1959). Later, Kelcham *et al* (1962) presented evidence correlating the sepsis rate after radical cancer surgery to the carrier status of the patients. Our previous studies of patients undergoing thoracic surgery confirmed this correlation. Thus, roughly 1/3 of those in whom postoperative staphylococcal sepsis developed were infected by staphylococci identical to those carried preoperatively in the anterior nares (Lindbom 1964, Lindbom & Laurell 1967). These reports indicate that endogenous staphylococci may be responsible for a variable proportion of septic complications in nasal carriers.

White (1961) showed by semi-quantitative studies that a correlation exists between skin carriage and heavy nasal carriage. Staphylococci could be recovered from only 5 per cent of skin cultures in patients who were carriers of less than 100 000 colonies per nasal swab, whereas the corresponding incidence in patients with more than 100 000 colonies per swab was 44 per cent.

On the assumption that the nasopharynx is the main human reservoir, its elimination as a source of infection would be a logical prophylactic measure to minimize the risk of autoinfection and, consequently, to reduce the over-all risk of sepsis. Delafield *et al* (1941)

nasal reservoir directly by local

Subsequently, various methods

action have been used, the com-

monest being a cream containing nystatin and chlorhexidine applied at intervals to the nostrils. Better results were obtained with a nasal spray containing gramicidin and framycetin (Stratford *et al* 1960).

methicillin ointment (Varga & White 1961) and by spraying the ward air with methicillin (Elek & Fleming 1960)

Nasal chemotherapy was therefore instituted in The Department of Thoracic Surgery as a preventive measure to be used by all patients. "Naseptin" was chosen for preoperative prophylaxis, whereas "Soframycin" was chosen for eradication treatment and for postoperative use, partly because it could be used in postoperative patients with a minimum of disturbance, in view of its mode of application.

The following four criteria were used to assess the efficiency of nasal chemotherapy: (1) Influence on the carrier rate of the population at risk; (2) Influence on the carrier state of individual patients; (3) Influence on the incidence of autoinfection; (4) Influence on the ecology of epidemic strains. Since they were parameters of different aspects of the host-parasite relation, they provided means of cross-validation.

MATERIAL AND METHODS

Characteristics of the patients, method of surveillance, antibiotic policy, arrangement of the wards and basic bacteriological technique have been described elsewhere (Lindbom *et al.* 1967). The epidemiological survey included extensive carrier examinations and standardized environmental studies in the intensive-care ward.

Bacteriological Routine

Nasal and throat swabs were taken from patients on the day of admission or as soon after as possible. They were also swabbed on the day of operation and daily during the postoperative phase in the intensive-care ward except on Saturdays, Sundays and Mondays. The study is based on totally 2736 nasopharyngeal cultures from which *Staph. aureus* was isolated on 90 occasions. The disc diffusion method was used for sensitivity determinations (Ericsson *et al.* 1954). All coagulase positive staphylococci were phage typed by the technique of Blair & Hill (1954).

Nasal Chemotherapy. On admission all patients received

septic cream (Naseptin®), 100%

50% 0.5 per cent. They were

times daily. In general practice

of the aim of the trial.

118 of 119 patients having been informed

All patients found to be carriers on admission, in addition to postoperative patients in the intensive care ward, were supplied with a nasal spray (Soframycin®) containing framycetin 1.25 per cent, gramicidin 0.005 per cent and metaxolone 2.5 per cent. The nebulizer was to be squeezed sharply into each nostril and the pharynx 3 times daily.

On their return to the nursing wards patients received a second tube of nasal cream.

A source of error common to all swabbing of patients undergoing nasal chemotherapy was the transfer of an antibacterial substance from the mucous membrane to the swab. This carry-over effect could not be excluded, but the risk was expected to be lowest immediately before the first treatment of the day. Consequently, when ever possible, nasopharyngeal samples were taken in the morning.

Definitions. Patients were classified as persistent carriers when more than three cultures were positive. They could be divided into two groups: those who carried the same phage type repeatedly, and those who changed types frequently. Persistent non-carriers were patients from whom more than three negative cultures were obtained. The remainder were classified as intermittent carriers.

Since both nose and throat swabs were used, the presence of staphylococci at either site was regarded as evidence of carriage at the time of swabbing.

BACKGROUND INFORMATION

The study took place from January 1st to December 31st, 1962, inclusive. Nasopharyngeal cultures were obtained from 402 patients, 313 of them had been operated on. Postoperative infection developed in a total of 58 patients. *Staph aureus* was isolated from septic complications in 41 (13.1 per cent) of these cases.

The pathogenesis of staphylococcal infections clearly depended to a considerable extent on host susceptibility. Infection attack rates ranged from 3 to 53.5 per cent. High rates were associated with multiple adverse factors. Taken as a group, operations on the heart and great vessels carried a higher risk of sepsis than pulmonary surgery. In the former, the overall rate was 22.9 per cent, ranging from 7.5 per cent after certain closed cardiac operations to 35.8 per cent after open heart procedures. Septic complications occurred in 12.5 per cent of the patients who underwent pulmonary operations. The infection attack rate was 27.3 per cent after pneumonectomy and 8.3 per cent after lobectomy.

Hospitalization in the unit lasted for 29.3 days for the "average" surgical patient, of this time he spent 8.5 days awaiting an operation lasting for 3.3 hours, and 4.5 days in the intensive care ward. Due to factors inherent in the patient/operating complex, there were marked variations in the duration of these phases (Lindbom et al 1967).

Data on phases of hospitalization, mode of nasal chemotherapy and nasopharyngeal examinations are summarized in Fig. 1.

Fig. 1
Phases of Hospitalization, Mode of Nasal Chemotherapy and
Nasopharyngeal Carrier Examination

Environment	Nursing ward	Intensive care ward			Nursing ward
Average duration	9.5 days	4.5 days			15.5 days
Nasal chemotherapy					
Non carrier	Naseptin	Soframycin			Naseptin
Carrier	Naseptin Soframycin	Soframycin			Naseptin
Carrier examination	↑ A	↑ O	↑ PO	↑ PO	↑ PO*

* Arrows indicate nasopharyngeal examinations on admission (A), at operation (O), and subsequently in the intensive care ward (PO).

RESULTS

Carrier Rate

On admission, a total of 142 out of 402 patients examined (35.3 per cent) were found to be nasopharyngeal carriers. A second culture was taken at operation, which implies that the carrier rate was studied in 85 per cent of the patients. The carrier rate was 41.4 per cent on admission and 26.7 per cent at operation. The degree of reduction was related to certain host factors such as age, underlying disease, previous hospitalization and antibiotic treatment. In 29 children, the carrier rate was reduced from 31.2 to 15.9 per cent. In 27 patients with pulmonary infection the rate fell from 43.7 to 17.7 per cent. In contrast to the children, the latter had been hospitalized for long periods before the current

admission owing to the severity and chronicity of the underlying illness. The majority had also received several courses of systemic antibiotic treatment.

Two preoperative and two postoperative cultures were collected from 146 patients, i.e. the influence of *prolonged* nasal chemotherapy (>2 weeks) was studied in 46.7 per cent of the operated population. In this group the rate of carriage was 41.8 per cent on admission, fell to 24.7 per cent at operation but rose to 43.8 per cent on the 2nd and 3rd postoperative day despite nasal chemotherapy. Later, the rate of carriage was established at nearly 50 per cent. It should be noted, however, that the number of cultures per patient was dependent on the time spent in the intensive care ward. Little information is therefore available about the carrier state of patients undergoing operations necessitating only a brief period of intensive postoperative supervision, whereas the bulk of information deals with patients on whom extensive operations had been performed. The former group including resection of aortic coarctation and lobectomy spent on average 2.3 days in the intensive care ward. This contrasted sharply to the latter group (replacement of diseased cardiac valves and pneumonectomy) who remained there for 6-11 days.

Antibiograms. Approximately 50 per cent of all nasopharyngeal staphylococci were resistant to penicillin and tetracycline. They were classified as hospital staphylococci. During the study 7.4 per cent of the strains tested were found to be resistant to neomycin.

Carrier State

Conclusions on the individual state of carriage in 146 patients were based upon sequences of four nasopharyngeal cultures.

Positive cultures on admission. This group consisted of 61 patients. In 16 of them staphylococci were also isolated from the three following specimens. In 10 patients the initial positive culture was followed by three negative ones. The remainder (30 patients) were intermittent. 11 yielded two positive cultures, 19 only one positive culture.

Negative culture on admission. This group consisted of 85 patients, 49 of whom remained negative. Ten patients were converted into a positive state and staphylococci were isolated from the three following specimens. Of the remainder (26 patients) 11 yielded two positive cultures and 15 only one.

In Table 1 the patients are classified on the basis of the number of positive cultures without reference to their order. It is seen that 45 (30.8 per cent) were persistent carriers, 22 (15.1 per cent) intermittent carriers and 79 (54.1 per cent) persistent non carriers.

Fragmentary evidence—based on the *trend* observed in sequences of 5 or 6 cultures obtained from patients in the intensive care ward for a prolonged period—showed that the proportion of persistently positive

carriers rose from approximately 30 per cent on the 2nd postoperative day to 50 per cent on the 5th. A large part of this increase could be traced to intermittent carriers.

Phage typing of staphylococci recovered from patients undergoing nasal chemotherapy permitted an analysis of its effect on individual carriers. As 11 cultures were regarded as a minimum sample, only 86 carriers were available for analysis. It was found that 33 per cent of the patients retained the strains present on admission whereas 22 per cent lost them. Hospital staphylococci were isolated transiently from 28 per cent and persistently from 17 per cent.

TABLE 1
Patterns of Carriage in 146 Patients as Determined by 4 Consecutive Nasopharyngeal Cultures

Classification	Pattern of carriage		No of		Sepsis rate %
	Positive	Negative	Patients	Infections	
Persistent carriers	4	0	16	11	68.8
	3	1	29	10	34.5
Intermittent carrier	2	2	22	4	18.2
Persistent non carriers	1	3	30	5	16.7
	0	4	49	11	22.5
			146	41	13.1

Relation between Carrier State and Rate of Sepsis

It is also evident from Table 1 that a positive correlation existed between the number of positive specimens and the rate of sepsis. It rose from 16.7 per cent in patients with one positive culture to 68.8 per cent in those with four. Obviously persistent non carriage did not preclude sepsis as postoperative infection occurred in 22.5 per cent of the patients from whom four negative specimens were obtained.

The rate of sepsis was 46.7 per cent in persistent carriers, 18.2 per cent in intermittent carriers and 20.3 in persistent non-carriers. To illustrate the clinical importance of the carrier state in 41 infected patients the acquisition of disease producing staphylococci is summarized in Table 2. Details of this epidemiological study will be presented in the following paper (Lindbom & Laurell 1967). Fourteen infections (34.2 per cent) occurring in persistent carriers were classified as endogenous. They were caused by strains carried on admission or required preoperatively in the ward. Twenty-seven infections were classified as exogenous. They occurred in all types of carriers. Four infections (9.8 per cent) were acquired in the theatre, 2 of 3 per cent in the intensive-care ward and 1 (2.4 per cent) in the ward.

Persistent carriers could be divided into two groups, i.e. 8 carriers of a single strain, and 12 carriers of multiple strains. Five of the latter

acquired their disease producing strains preoperatively 1 during surgery and 6 postoperatively in the intensive care ward. In persistent non-carriers 2 infections were theatre acquired and 14 ward acquired.

TABLE 2
Summary of Data in 51 Cases of Staphylococcal Sepsis

State of carriage	Acquisition of disease producing staphylococci				Ward
	Before admission	Ward	Op theatre	Intensive care ward	
Persistent (single strain)	3				9
Persistent (multiple strains)	3	2	1	6	12
Intermittent			1	3	4
Non carriage			2	13	15
	12	2	4	22	1

— endogenous infection
 — exogenous infection

Taken as a group persistent carriers were comparable with regard to patterns of carriage although they differed individually with respect to strains carried and various patient factors. Analysis of carriers and non-carriers infected and non-infected revealed that patients in whom sepsis developed had generally undergone extensive surgical procedures such as certain types of open heart operation and pneumonectomy that were associated with multiple adverse factors. Thus infection prone persistent carriers tended to contract endogenous infections whereas persistent non-carriers contracted exogenous infections when exposed to certain strains prevalent in the environment.

DISCUSSION

The effects of nasal chemotherapy reported by various authors have been difficult to evaluate in view of the lack of generally accepted criteria. The index of success has often been calculated from the difference between pre- and post-treatment cultures. Intermittent carriers sometimes occurring in up to 60 per cent of a population (Hutchinson *et al.* 1957) can be expected to give rise to a certain number of false negative results. In the hands of Bassel *et al.* (1963) the reliability of a single swab was 80 per cent. This implies that in the absence of frequent swabbing intermittent carriers might be incorrectly classified as successfully treated. Persistence of staphylococci in the nose despite chemotherapy must also be differentiated from the acquisition of new strains. It might have been ideal if the carrier state of patients

undergoing treatment had been known before it was started, but this was not practicable in the present study. Instead, the pattern of carriage had to be determined during therapy. The use of controls was ruled out because the nasal carrier state was unknown, and the population was too small and too heterogeneous for its variations to be equalized by random sampling.

In patients who were to be operated on, the carrier rate on admission was 41.4 per cent, i.e., well within the 35–50 per cent range normal for adults. Although hospitalization generally increases the proportion of persons carrying hospital staphylococci and tends to produce an overall rise, nasal chemotherapy actually reduced carriage during the pre-operative phase by about 40 per cent. This initial decrease was counter-balanced within 1–2 days of operation by an equal increase, despite continued treatment. There are several possible explanations of this unexpected rise. The simplest one is the presence of neomycin-framycin-resistant strains which rapidly colonized postoperative patients. This was not, however, the case as only a fraction (7.4 per cent) were resistant. Alternatively, Soframycin spray may have had a less protective effect than Naseptin cream, although this would be at variance with the results of *Stratford* and co-workers (1960), who found Soframycin to be superior to Naseptin and claimed 100 per cent success in treatment. It is conceivable that nasal chemotherapy was, of necessity, less than adequately applied during the early, hectic postoperative phase in patients with urgent circulatory or ventilatory problems. This possibility cannot be disregarded. Yet, the disproportionate frequency of persistent non-carriers in the population at risk (50 per cent) seems to be attributable to fairly strict nasal chemotherapy.

Since nasal chemotherapy caused a preoperative decrease in the over-all carrier rate, but failed to prevent a postoperative increase, it is obvious that environmental conditions differed.

The epidemiological study, of which the results will be reported in the following paper (*Lindbom & Laurell 1967*), showed that certain patients—disseminators—caused massive, widespread environmental contamination in the intensive-care ward. This ward was a bottle-neck, where newly operated patients in close proximity were often exposed to massive doses of disseminated staphylococci. In contrast, only a fraction of newly admitted patients were exposed to disseminators in the nursing ward.

Nasal chemotherapy had little effect on the individual pattern of carriage. It did not materially reduce the proportion of persistent carriers, which remained at about 10 per cent. Phage typing and antibiograms revealed that 22 per cent of the patients lost staphylococci carried on admission while 33 per cent found them. The rapid post-operative increase in the carrier rate occurred mainly in intermittent carriers. Hospital staphylococci were isolated transiently from 20 per cent of the patients and persistently from 17 per cent.

The present results support the view of Williams *et al* (1959) that a relation exists between staphylococcal carriage and an increased risk of sepsis. The rate of sepsis rose from 16.7 per cent in patients with one positive culture to 68.8 per cent in those with four. Sepsis was 3 times as common in persistent carriers as in persistent non carriers. Obviously, no direct causal relation existed since sepsis developed in 1 of 5 non carriers.

A characteristic of the population at risk was that endogenous staphylococci accounted for 34.2 per cent of all infections. They were particularly common in patients on whom valve replacement operation had been performed and were responsible for every second case of sepsis (Lindbom *et al* 1967). Thus, the risk of sepsis was dependent not only on the carrier status but also on numerous factors inherent in the patient and his operation, such as underlying disease, functional state of heart and lungs, type of surgical procedure and postoperative tracheostomy.

But it was equally obvious that environmental factors were of importance (Lindbom & Laurell 1967). Exogenous staphylococci acquired in the intensive care ward accounted for 31.3 per cent of all postoperative infections. Consequently, staphylococci carried by the patient at operation or acquired postoperatively caused 85.5 per cent of all infections.

SUMMARY

Under the existing conditions nasal chemotherapy neither eradicated nor suppressed staphylococci carried persistently in the nasopharynx. Nor did it protect intermittent carriers from the implantation of virulent hospital staphylococci. The ecology could not be influenced by this measure, and the incidence of infection was not reduced.

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STUDIES ON THE EPIDEMIOLOGY OF STAPHYLOCOCCAL INFECTIONS

5. Importance of Environmental Factors During the Early Postoperative Phase

By

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In a previous study which dealt primarily with clinical aspects, intra-thoracic operations were found to be associated with a wide variation in the specific infection attack rate which ranged from 0-53.5 per cent (Lindbom *et al.* 1967). In a heterogeneous population the incidence of postoperative sepsis showed a positive correlation to numerous patient factors, such as underlying disease, functional state of heart and lungs, type of surgical procedure, postoperative tracheostomy, indwelling catheters in the superior vena cava and multiple drainage tubes. Such factors were primary determinants of whether a given staphylococcus in a given patient was to cause disease. Infection-prone cardiac patients were found to contract sepsis even when staphylococci of known virulence were absent from the unit. Indeed, there were outbreaks of non-epidemic staphylococcal disease caused by endogenous staphylococci. The importance of the staphylococcal carrier state has been described separately in Part 4 (Lindbom & Laurell 1967). Sepsis developed in about 50 per cent of all persistent carriers who were in need of prolonged postoperative supervision because of circulatory and respiratory problems. But it was equally obvious that concurrent environmental factors, such as structure of the unit, turnover and intramural movement of postoperative patients contributed to the creation of a common source reservoir where patients were exposed to staphylococci that varied continuously both quantitatively and qualitatively.

To determine the influence of environmental staphylococci on the relation between a patient and his surroundings, studies were undertaken to determine *when* and *where* pathogenic staphylococci were acquired by patients in whom sepsis developed and *how* epidemic strains were disseminated.

MATERIAL AND METHODS

Characteristics of the patients, definition of terms, method of surveillance, antibiotic policy, nasal chemotherapy, arrangement of the wards and basic bacteriological technique have been described elsewhere (Lindbom *et al* 1967). An account is given in the following of a comprehensive epidemiological survey, including regular carrier examinations, standardized environmental examinations in the intensive care ward and a pilot study of the operating theatre.

Environmental investigation. (a) Intensive care ward. Standardized studies were performed each week from Tuesday to Friday inclusive. They were focused on the patients and their immediate surrounding. Consisting of 6-10 cultures, depending on the number of patients in the ward, each 'patient-environment unit' included sampling of the nasopharynx, bedding, furniture, equipment, air and personal belongings.

The 'sweep plate method' was used for sampling of bedclothes and other fabrics. Bacterial contamination was sampled by rubbing the relevant surface with sterile cotton swabs wetted with broth. 'Sedimentation plates' were used to examine simultaneously the air from several sites in the ward. Standard Petri dishes con-

of the operating team. At each operation, 10 plates were placed around the patient at predetermined sites and exposed for the duration of operation.

environment units collected on 224 different days. In addition, 267 nasopharyngeal cultures were obtained from 77 members of the staff and personnel. The study is based on a total of 2670 staphylococcal isolates specified in Table 1.

TABLE 1

Background Information on Staph aureus Isolated in the Thoracic Surgical Unit

Sample obtained	Source	Material	No of strains
Routine work up	Patient	Clinical specimens	
		Wound infections	85
		Blood	23
		Faeces	13
		Sputa, bronchial secretæ	89
			210
Epidemiological study	Patient	Nasopharyngeal cultures	905
	Environment	Air/culture plates	659
		Contaminated objects	204
	Personnel	Nasopharyngeal cultures	92

BACKGROUND INFORMATION

Detailed information about the patients, their operations and infection attack rates was given in previous papers in this series (Lindbom *et al*, Lindbom & Laurell 1967). These also contained data on phage patterns and antibiograms of disease-producing staphylococci. Consequently, this information relevant to the epidemiological approach will be given here.

The total number of operated patients was 313. The monthly average of operations per operating day varied from 1.1-1.3, exceptions being May-July, September and

December when it reached 17. The duration ranged from 12.96 hours (average 33 hours). The average patient remained 29.5 days in the unit; the preoperative phase lasted for 9.5 days; the postoperative supervision phase spent in the intensive care ward for 4.5 days. Due to patient factors the duration of these phases varied considerably. The need of supervision in particular was greatly influenced by patient/operation factors. It ranged from 24 hours to 52 days.

The intensive care ward was a small area divided into three communicating parts: two 11-bed sections and one single bed emergency room. There was a constant need for isolation facilities in this open ward which was the centre of the unit of postoperative supervision and specialized intensive care. Highly susceptible patients with circulatory and ventilatory problems were nursed in close proximity for long periods. In practice it functioned as a bottle neck through which most newly operated patients had to pass and affected the turnover of patients.

Variations in the operating list influenced the population in the intensive care ward. When several short operations were performed in one day, the need of postoperative supervision was generally short. The turnover of postoperative patients was rapid. When several open heart operations were concentrated to a brief period, turnover grew slow, resulting in an accumulation of infection prone patients. Consequently there were periods when the population at risk was composed of patients with low to moderate susceptibility, alternating with periods when most patients were characterized by high susceptibility. Exposure to the ward lasted for 1-3 days for low risk patients and 10-13 days for high risk patients.

There were appreciable variations from month to month in number and types of operation. Open heart surgery comprised 12.56 per cent of all operations, averaging 33 per cent in October and November; it made up 48 and 56 per cent respectively. No direct relation could be discerned between fluctuations in the monthly rate of sepsis and total number of operations or their sum in hours.

In early January when the study was started, staphylococci of proven virulence—mainly strains 75/77 and variants of the 81 complex—were not uncommon in the nasopharynx of patients and personnel. 15 per cent of the former, including 1 disseminator of 75/77 staphylococci, and 4 per cent of the latter were carriers. Total summer closure in July almost completely disposed of such strains as they were found in less than 1 per cent of both patients and personnel in August.

Classification of infections. Infections were classified as endogenous or exogenous. When a patient who was a carrier of a particular strain on the day of operation subsequently contracted a septic lesion, it was clearly impossible to determine whether contamination actually took place in the theatre or in the wards, but for epidemiological purposes classification of the infection as of endogenous origin was equally relevant.

Infections were defined as *exogenous theatre acquired* on the basis of the following criteria: if a known nasopharyngeal carrier participated in the operation or was present in the "critical area" of the theatre, the lesion was diagnosed within 5 days of operation and the disease producing strain was identical to the carrier strain. Other sources were disregarded except in a pilot study of 18 open heart operations that were monitored by sedimentation plates and nasopharyngeal cultures from all members of the team.

If no known nasopharyngeal carrier of a strain that caused postoperative sepsis was present in the theatre and clinical bacteriological evidence implied that the strain had not been acquired until after operation, contamination was assumed to have taken place postoperatively. Such cases were denoted as *endogenous or exogenous ward acquired infections*.

RESULTS

Epidemiology of Staphylococcal Disease

Postoperative infection developed in a total of 58 patients. *Staph aureus* was isolated from septic complications in 41 of these. Information about certain aspects of the situation is summarized in Fig. 1. The monthly incidence of staphylococcal sepsis ranged from 0.296 per cent, with a cumulative incidence of 13.1 per cent.

Up to summer closure, 19 of 33 infections were due to staphylococci. The maximal incidence, 17.2 per cent, was reached in May, when 5 open heart patients contracted sepsis, 3 were endogenous infections. As a whole, the situation was characterized by numerous cases of sporadic infection. Only 7 septic cases could be attributed to endemic staphylococci, 75/77 and a strain denoted as "Thorax BP" (for phage pattern, see Lindbom *et al* 1967), which had been present in the unit for prolonged periods without creating epidemic conditions. Excluded from Fig. 1 were 6 cases of ill defined respiratory tract symptoms associated with slight fever and copious sputum from which 75/77 staphylococci were recovered.

After summer closure, the staphylococcal sepsis rate rose to a maximum of 29.6 per cent in November. Twenty-two of 25 infections were caused by staphylococci. Two strains, 75/77 and "Thorax I" (for phage pattern see Lindbom *et al* 1967), accounted for 15 infections, while sporadic infections made up a smaller proportion. Whereas Thorax I was present continuously over a long period, 75/77 staphylococci were repeatedly introduced into the unit by carriers from other parts of the hospital, in whom endogenous infections developed.

Partial closure in late December reduced the rate of sepsis to 12.5 per cent.

Sources of Staphylococcus

Staphylococcal Carriers Patients. The over-all carrier rate on admission was 35.5 per cent. In a group previously hospitalized for long periods due to the severity and/or chronicity of the underlying illness, it was 41.8 per cent. About 15 per cent of the former and 45 per cent of the latter carried staphylococci resistant to both penicillin and tetracycline.

Nasal chemotherapy was used throughout the study in all patients. As a detailed account of its effect will be published separately, results will be summarized briefly.

Nasal chemotherapy resulted only in a temporary preoperative reduction of 40-50 per cent in the carrier rate. Within a few days of the operation the rate had returned to the level on admission. Postoperative changes in the nasopharyngeal flora were studied by repeated swabbings. In this selected group of long exposure patients about 33 per cent retained strains carried on admission. Hospital staphylococci acquired before and after operation were recovered from 16 and 55 per cent respectively. The latter were a transient finding in about 2/3 of the patients and persistent in 1/3. The incidence of consistently positive carriers rose from 33 per cent on the second postoperative day to 50 per cent on the fourth. As the need of prolonged supervision was closely related to the type of surgery, colonization was most common in patients with a valvular prosthesis.

Personnel. The over-all carrier rate as determined in 77 persons was 34.6 per cent. Repeated nasopharyngeal cultures were obtained from 44 members of the permanent staff. Of these 25 per cent were persistent carriers, 34 per cent intermittent carriers and 41 per cent non carriers.

TABLE 2

Relative Incidence of Environmental Staphylococcal Strains Isolated in the Intensive Care Ward

Phage pattern	Relative incidence %											
	Jan	Feb	March	April	May	June	July	Aug	Sept	Oct	Nov	Dec
80/81 complex	1	5	7	17	13	1			10	1	2	1
6/7/47/53/75	12	2	14	2	9	20			7	5	2	3
75/77	34	53	6			1			5	1	15	2
RTD × 1000 6/7/47/53/75/77	7	1	17	20	6	1			8	7	5	9
RTD × 1000 Thorax BP	1	1	1	15	15	44			20	16	4	2
RTD × 1000 Thorax I										19	47	40
RTD × 1000 NT	17	11	24	13	16	18			12	14	10	13
Various other patterns at RTD × 1000	27	19	21	16	14	14			17	8	6	17
Total incidence of <i>Staph aureus</i> typed at RTD × 1000	52	32	63	64	54	76			12	65	72	81
Total monthly no. of positive environmental isolates	35	75	66	88	96	46			110	69	199	74

The following staphylococcal strains were isolated from persistent carriers: (a) physicians Thorax BP RTD × 1000 29/80/56 and RTD × 1000 52/56 (b) nurses 3A 75/77 and 6/7/47/53/54/75 (c) nurses 81/56 3A Thorax BP (d) technicians RTD × 1000 53/42D

As about 23 of the personnel were persistent or intermittent carriers strains prevalent in the environment were frequently recovered from their nasopharyngeal swabs. Heavy carriage was however uncommon and in about 34 enrichment cultures were necessary to recover staphylococci.

The surgical team comprised no nasopharyngeal carriers of staphylococci belonging to the 52/56/80/81 complex or characterized by the 75/77 or Thorax I pattern. With the exception of strains Thorax BP and RTD × 1000 53/42D no staphylococci in the personnel could be matched with strains subsequently responsible for sepsis.

Environmental Pool Operating theatre A pilot study of the theatre environment was performed during 18 heart operations from October to December inclusive. Altogether 32 colonies of *Staph aureus* giving 8 distinct lytic patterns were recovered from sedimentation plates. Three strains could be matched with staphylococci carried by persons present: one strain Thorax BP was carried by 2 surgeons, the other two were patient strains.

Intensive Care Ward Results of standard environmental studies are summarized in Table 2. It shows the monthly incidence of some common strains as well as an estimate of the intensity of contamination. Whereas the level of non-typable staphylococci remained fairly close to 10 per cent throughout the year most strains showed marked cyclic

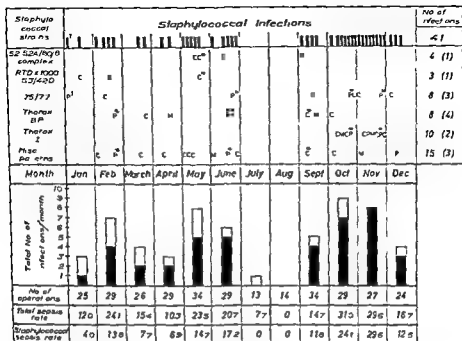


Fig 1

- C Cardiac operation single strain of *Staph aureus*
 C* Cardiac operation several strains of *Staph aureus*
 P Pulmonary operation single strain of *Staph aureus*
 P* Pulmonary operation several strains of *Staph aureus*
 II Miscellaneous operations
 Bracketed figures denote mixed staphylococcal infections
 ■ *Staph aureus* infections
 □ Other bacterial infections

¹ Operation performed in 1961 not included

variations, with steep increases and decreases. The build up phase either preceded, coincided with or followed an accumulation of postoperative infections presented in Fig 1, depending upon such variables as degree of dissemination and average length of incubation period.

Staphylococci characterized by the 75/77 pattern dominated the picture in January-February, when they comprised 34-53 per cent of the pool. Another rise, to 15 per cent occurred in November. During April-June, a second strain, Thorax BP emerged as a common contaminator, reaching a maximum of 44 per cent in June. From 25 per cent in September it fell slowly to a few per cent. It was replaced by a third strain Thorax I, which dominated the pool in October-December, reaching a maximum of 47 per cent in November.

Staphylococci belonging to the 52 52A/8Q/81 complex formed a variegated group which comprised a small fraction of the pool during the greater part of the year. Only in April-May and September did the level exceed 10 per cent.

TABLE 3

Classification and Comparison of Staphylococci from Different Sources by Phage Typing

A Patient nasopharyngeal strains

Group	Phage RTD	Dilution RTD \times 1000	No of strains	%
I	81	67	148	16.4
II	53	14	67	7.4
III	225	178	401	44.5
I+III		244	214	23.7
NT		73	73	8.1
No of strains	359	546	905	
%	39.7	60.3		

B Personnel nasopharyngeal strains

Group	Phage RTD	Dilution RTD \times 1000	No of strains	%
I	20	33	54	20.7
II	15	16	31	11.7
III	38	64	102	38.4
I+III		58	78	21.8
NT		22	22	8.3
No of strains	73	193	266	
%	27.5	72.6		

C Environmental strains

Group	Phage RTD	Dilution RTD \times 1000	No of strains	%
I	51	24	75	8.7
II	41	22	63	7.3
III	256	130	386	44.7
I+III		251	251	29.0
NT		88	88	9.8
No of strains	348	515	863	
%	40.7	59.2		

Conformity between Different Sources

By way of comparison phage typing results relating to nasopharyngeal strains obtained from patients and personnel in addition to environmental strains are summarized in Table 3. They have been arranged by phage group and phage dilution.

There was a close conformity in phage group distribution between staphylococci recovered from patients and their environment with the

single exception of group I staphylococci which were twice as frequent in the nasopharynx as in the surroundings. This is in accordance with the observation that individuals whose strains changed were recolonized with such organisms as appear in the greatest number. Although the general distribution of strains carried by the personnel was similar to that of patients it should be noted that groups I and II staphylococci were slightly more common among the personnel—who were not receiving nasal chemotherapy—than groups III and I + III which were less common. The distribution showed relatively good conformity between all three sources only when strains were characterized by high indices of contamination e.g. groups III and I + III.

Time and Place of Acquisition of Disease Producing Staphylococci

Endogenous Infections Autoinfections due to endogenous staphylococci developed altogether in 14 patients (34.2 per cent). 10 were due to strains carried in the nasopharynx on admission, 2 were acquired in the unit before operation and the remaining 2 were isolated preoperatively from superficial skin pustules but not from the nasopharynx. Phage typing disclosed that group III strains had produced 8 infections including 3 cases of 75/77 sepsis, group II strains 4 infections and group I and group I + III strains one infection each.

Exogenous Theatre Acquired Infections During the pilot study 7 patients contracted postoperative sepsis. Only one infection, a septic wound from which *Staph aureus* (Thorax BP), *Staph albus* and *E coli* were isolated, fulfilled the criteria set up. The others were either autoinfections or due to strains not present in the theatre e.g. Thorax I.

During the whole year only 4 cases (9.8 per cent) were classified as exogenous theatre acquired. Thus in addition to the Thorax BP infection mentioned there were 3 infections all due to staphylococci with the RTD $\times 1000$ 53-42D pattern which fulfilled the criteria. There was only one known source of this strain in the unit, a technician who was present during open heart operations subsequently complicated by infection.

Endogenous or Exogenous Ward Acquired Infections The average interval between operation and recognition was 13 days. Roughly 75 per cent of all infectious complications were diagnosed within 15 days, 66 per cent between the 5th and 15th day.

Intensive care ward Since the number of carriers increased with the time spent in this ward and the need of prolonged intensive supervision was closely related to the type of surgery, nasal colonization was most frequent in patients who had undergone open heart surgery or pneumonectomy. Strains identified as 75/77 Thorax BP and Thorax I were isolated from the nasopharynx of 63 patients. It could not be established whether nasal colonization actually preceded wound contamination and caused endogenous infection, occurred simultaneously

with inoculation of tissues by exogenous staphylococci, or was secondary to a septic complication. For epidemiological purposes, the classification of 22 infections (53.7 per cent) as ward-acquired sufficed.

The incidence of postoperative sepsis was 50 per cent in long-exposure patients from whom consistently positive nasopharyngeal cultures had been obtained, as compared to 18.8 per cent in consistent non-carriers.

Nursing ward The epidemiological importance of the nursing wards was insufficiently studied. Only one open-heart patient—a non-carrier on discharge from the intensive-care ward on the 8th day—was believed to have acquired the contaminating 75/77 strain there. It caused wound sepsis on the 16th day. There was no known carrier among the personnel.

Ecology of Staphylococci in the Intensive-Care Ward

The pool of staphylococci in the intensive-care ward was analysed by regular environmental studies. The weekly number of distinct strains recovered from 'patient-environment units' was 5–15. They were usually present during short periods (2–3 days), which corresponded to the duration of intensive care for the average patient. Only a few strains persisted in the ward.

Relation between Patient and Inanimate Environment

A strain found in the nasopharynx of a patient could only rarely be matched with staphylococci recovered from his inanimate environment in the theatre or intensive-care ward.

Although staphylococci generally could be isolated from a limited area surrounding an infected patient, contamination was rarely heavy as long as the lesion could be covered with adequate dressings. Heavy contamination frequently accompanied cases of staphylococcal diarrhoea with profuse watery stools or a septic tracheostomy wound, with or without complicating tracheobronchitis and bronchopneumonia. The presence of a disseminator was signalled by heavily contaminated air, bedding and floor. Within a day or two the strain was widely dispersed, often isolated in the proximity of several non-carriers, some of whom subsequently became nasal carriers. The ward continued to be heavily contaminated until the patient was removed, when the strain disappeared within a couple of days.

Of special interest were staphylococcal strains characterized as 75/77 Thorax I and Thorax BP. Their epidemiological behaviour and ability to cause infection will be described in some detail.

Strain 75/77 This strain was an easily identifiable 'marker' organism which has been endemic in many parts of the University Hospital since 1961 (Lindbom 1964). Characterized by a clear-cut 75/77 pattern both at RTD and RTD \times 1000, strains from various parts of the hospital

differed mainly with respect to their resistance to tetracycline. Despite partial closure of the unit around the turn of the year 1961-62, they remained firmly entrenched there.

In December 1961, pneumonectomy had been carried out in a man with bronchial carcinoma. The postoperative course was complicated by a bronchial fistula, empyema, and diarrhoea. Thoracoplasty on January 10th failed to arrest the infection. Bronchopneumonia and respiratory failure ensued. 75/77 staphylococci were isolated in large numbers from the sputum, bronchial secretion and faeces during a period of 35 days. He remained in the intensive-care ward for 8 days after the second operation. Being a copious disseminator, 75/77 staphylococci were recovered from his bedclothes, objects in his immediate environment, and at some distance away in the proximity of other patients, who were either non-carriers or carriers of other strains. The 75/77 staphylococci were present *continuously* in the ward from the middle of January until the first week of March. As shown in Table 2, they comprised roughly 34 per cent, 53 per cent and less than 10 per cent of all positive environmental samples during this period.

No other cases of wound infection attributed to strain 75/77 occurred during this period of heavy environmental contamination. In June, a septic wound, classified as an auto-infection, developed in a patient who was a carrier of the strain already on admission. Colonization of the respiratory tract with 75/77 staphylococci was, however, a frequent phenomenon causing ill-defined infection of the respiratory tract or symptomless carriage. During the 8-week period when 54 patients were exposed to this massively contaminated environment, 14 became colonized despite nasal chemotherapy. With few exceptions, these patients had been tracheotomized and had remained twice as long in the ward as the others (>7 days). Six patients subsequently ran a slight temperature, with concomitant profuse mucopurulent expectoration, from which 75/77 staphylococci were isolated. One patient—initially a non-carrier—on whom cardiac surgery had been performed, had purulent sputum and diarrhoea. Various postoperative complications necessitated a prolonged stay in the intensive care ward (31 days), where he was the main 'disseminator'. After his discharge in March, the strain rapidly became uncommon.

In the autumn 75/77 staphylococci were comparatively unusual in environmental cultures, with a relative incidence of 5 per cent or less, except in November, when 15 per cent of all staphylococci belonged to this strain. Nevertheless the risk of infection is higher than in the spring.

The first postoperative infection occurred in September. A pneumonectomy performed on September 1st was complicated by a bronchial fistula. Six nasopharyngeal cultures obtained on admission and regularly afterwards, had been free of staphylococci. On the 15th, 11 days after the first reoperation, staphylococci were recovered for

the first time from the patient's nasopharynx. At this time, the only known source in the whole unit including the operating team, was a nurse, a healthy persistent carrier of the strains for more than 1 year. During the early postoperative phase she had helped to remove secretions from his airways by means of suction catheters. Not until the 17th, when the strain was found in copious amounts of tracheobronchial secretions, could it also be recovered from the patient's immediate environment. As he did not survive a second re-operation, performed 2 days later, the period of dissemination was short. In tissues removed at autopsy, 75/77 staphylococci and *E. coli* were present in large quantities. Within 2 days of his death, 75/77 staphylococci were no longer recovered in the ward. During a period of 4 days, when the strain had been present in the air and on objects in the immediate environment of this tracheotomized patient, 12 patients were exposed to it. Single colonies were recovered from sedimentation plates in the vicinity of 3 patients, a few colonies were recovered once in a single culture from the nasopharynx of one.

In October-November, 75/77 staphylococci were responsible for post-operative sepsis in 4 patients. Since 2 had been carriers of identical strains on admission, their infections were classified as auto-infections of the remaining 2 patients, 1 had acquired the strain in the ward before operation and 1 in the intensive care ward afterwards. All patients had been tracheotomized, but only 2 began to disseminate staphylococci within a week of operation. As they remained in the ward for only a short period thereafter, contamination was discontinuous and not extensive. In total, 50 patients were exposed to 'showers' of 75/77 staphylococci. The strain was isolated from sedimentation plates in the environment of 15 patients, and transiently from the nasopharynx of 5 patients.

In December, a wound abscess from which 75/77 staphylococci were recovered appeared in one patient. The diagnosis of septicaemia was considered but blood cultures remained negative. The patient had acquired the strain after operation, presumably from an unknown source in the nursing ward.

Strain Thorax I (RTD $\times 1000$) This strain was not present in the unit until October 15th, when a carrier with ruptured chordae tendineae was admitted. Open heart surgery was performed 2 days later. Owing to complications mainly of a non-infectious nature he had to remain in the intensive care ward for 54 days. A trivial infection of the tracheostomy wound and of the sternal incision developed on the 5th and 8th postoperative day, respectively. Strain 'I' was recovered from a total of 121 samples obtained during 28 separate environmental studies, it was isolated from the nasopharynx, bronchial secretions, his bedclothes, objects in the immediate surrounding, and the air.

Being continuously present in the ward Thorax I strain was also recovered in the immediate environment of 28 patients all of whom

were non carriers or carriers of other staphylococci at the time of admission. In October this strain was found in 19 per cent of all positive samples, in November in 47 per cent and in December in 40 per cent. Of 63 patients exposed to it in the intensive care ward 13 subsequently became colonized. The time they spent there ranged from 2-10 days (average just over 6 days). Sepsis subsequently developed in 9 patients including 3 with a mixed 75-77 and Thorax I infection. Again tracheostomy was the common denominator present in 7 patients. Only 2 infections were trivial. Septicaemia was suspected on clinical grounds in 3 patients, all of whom died; in 2 the diagnosis was confirmed at autopsy. Empyema developed in the 2 remaining patients. One patient with a perfectly healed wound after Beck's operation contracted staphylococcal diarrhoea.

Strain Thorax BP (RTD $\times 1000$) This was a fairly common organism in the intensive care ward (Table 2) and was recovered from the nasopharynx in 22 of 73 patients who had been exposed. Heavy carriage was rare. Over the period of investigation the antibiograms gradually changed. Initially Thorax BP staphylococci were sensitive to antibiotics with the exception of penicillin. In April variants resistant to tetracycline and chloramphenicol were noted together with sensitive strains. Several months later sensitive strains had all but disappeared. Obviously it cannot be stated unequivocally that strains from various human and inanimate sources were identical because of similarities in a common basic pattern. Nevertheless there was a causal and temporal relation between several consecutively infected patients and a similarity in disease pattern that suggested the possibility of cross infection.

In 8 patients trivial to moderately severe infections were diagnosed generally in the form of septic wounds. They yielded a mixed flora of various staphylococcal strains including strain Thorax BP. *Staph. albus* and/or enteric gram-negative bacteria. Characteristically Thorax BP was a secondary invader which did not cause a single case of monobacterial infection. There was one suspect case of septicaemia and none of diarrhoea. Although isolated from sputa and bronchial secretions in several patients these were neither copious nor markedly purulent.

Most of the time there was no recognizable source of Thorax BP staphylococci in the unit. It was isolated only transiently from the nasopharynx of patients and personnel or recovered sporadically from air and inanimate objects. There were however 5 episodes of increased environmental contamination traceable to human sources. In February cultures yielding the BP strain were obtained from the blanket of a patient who had undergone surgery for a traumatic rupture of the trachea 5 days earlier. Within a few days it was recovered together with another staphylococcal strain from sputa and purulent wound secretions. Environmental contamination was insignificant. A few weeks later it had colonized the skin of a patient with exacerbation of atopic dermatitis. Appropriate dermatological treatment cleared up the

skin condition, and the postoperative course after pericardectomy was uneventful. He nevertheless remained a moderate disseminator to bedding and ambient air. A trivial sternal wound infection from which Thorax BP staphylococci and *E. coli* were recovered subsequently developed in a tracheotomized fellow patient with a Starr prosthesis. The upper respiratory tract was also colonized but he did not become a disseminator. A third patient was colonized 2 weeks later, a few days after closure of an oesophago-bronchial fistula. Repeated cultures of his sputa yielded BP staphylococci, but no septic complications appeared and environmental contamination was slight. Throughout May the BP strain was recovered sporadically from sedimentation plates, but there was no known human source. In June, a tracheotomized open-heart patient acquired the strain and became a moderately heavy disseminator, but his postoperative course remained uneventful. Trivial wound infections later appeared in two pneumonectomized patients present in the ward. In September, a minor auto infection occurred in an open-heart patient. Environmental contamination was insignificant, but there was a second infection following excision of an aortic aneurysm in a patient who stayed simultaneously in the ward. In October, one source of Thorax BP staphylococci could be traced to a non-infected open-heart patient, who carried the strain before operation. It was recovered sparingly from his nasopharynx, bedding and ambient air.

DISCUSSION

The whole of 1962 was characterized by a fairly high incidence of staphylococcal disease, i.e., 41 cases (13.1 per cent). The monthly rate ranged from 0 to 30 per cent; for 5 months it was <10 per cent, for another 5 months 10–20 per cent, and for 2 months 20 per cent. The absence of septic complications occurred during the fortnight that preceded summer closure in July and that which immediately followed it in August, when the operating team was not complete and only short operations were performed. There were no open heart operations and a single pneumonectomy. The epidemiological importance of postoperative staphylococcal sepsis is obvious from the fact that it accounted for between 50 and 100 per cent of all infections during 9 months of the year—it made up 66 per cent or more during 6 months.

Most well defined infections were sporadic during the earlier part of the year. Although short outbreaks of postoperative sepsis occurred most cases were due to strains causing single infections. They seemed to bear a closer relation to a periodic accumulation of infection prone patients than to the presence of epidemic staphylococci. The latter part of the year appeared to differ, since two strains 75/77 and Thorax 1 gave rise to numerous infections. Despite these variations important epidemiological similarities were observed.

An earlier paper described the influence of hospitalization on patients

as reflected by changes in the carrier rate and the carrier state (Lindbom & Laurell 1967). An important selective factor was the routine use of prophylactic antibiotics. Given pre- and/or postoperatively they determined which types of staphylococci formed the hospital reservoir. At one time or another, approximately 60 per cent of the patients harboured strains resistant to penicillin, tetracycline and chloramphenicol. Patients receiving prophylactic antibiotics—either in large doses or in combination—acquired prevalent, multiresistant staphylococci with striking frequency and rapidly despite nasal chemotherapy. This is in agreement with the results of *Louria & Kaminsky* (1962), who demonstrated that the frequency with which patients harboured hospital staphylococci was related to both the kind and the amount of antibiotics administered. Actually, a combination of nasal chemotherapy and systemic prophylaxis failed to protect postoperative patients from either endogenous or exogenous staphylococci.

As to the origin of infecting staphylococci, they could be traced to human sources in 32 of 41 cases, whereas in 9 it remained unknown. The hospital staff seemed to play a less significant role in 1962 than in 1961 (Lindbom 1964). Only 5 patients were found to be infected by strains isolated earlier from personnel with whom they had been in contact. It is recognized, however, that nasopharyngeal swabs, obtained once every second month, fail to reveal carriage elsewhere (e.g. axillae, perineum, faeces, boils on unexposed skin) and may be insufficient, particularly in individuals who were intermittent carriers. Since the ability of nasal carriers to disseminate staphylococci seems to depend largely on high nasal counts (*White* 1961), the fact that it was necessary to use enrichment cultures to recover staphylococci from 34 of the staff suggests that their ability was low. Nonetheless, unknown sources among the staff may have been responsible for one or more of the 9 cases in which the origin of the infecting strain could not be ascertained. Altogether 27 infections could be attributed to staphylococci originating from patients. Frequent swabbings reduced the probability that unknown patient sources accounted for these additional infections.

Endogenous infections comprised more than 13 of the infections (14 cases). The infecting strain was isolated from the nasopharynx of 10 patients on admission and from skin pustules of 2 patients in the ward. Another 2 patients became nasopharyngeal carriers before operation. This experience emphasizes the clinical importance of the association between nasopharyngeal carriage and postoperative sepsis described by *Williams et al* (1959). This is not, however, an universal experience, probably because of differences in hospital operation factors. Tissue contamination may have taken place either in the theatre or postoperatively.

Of 27 exogenous infections only 14 are classified as theatre-acquired, i.e., they could be ascribed to staphylococci carried by persons present in the theatre at operation. Unfortunately, sources other than naso-

pharyngeal carriers were disregarded, except during a pilot study of 18 open-heart operations, when the theatre was monitored by sedimentation plates. Although results of a more extensive bacteriological work-up indicated that theatre-acquired infections were relatively rare, late septic complications may have been seeded by unknown carriers among the theatre staff. That this must be regarded as a distinct but unproven possibility is also evident from an analysis of infections regarded as acquired outside the theatre.

By definition, 23 exogenous infections were classified as ward-acquired, 22 were probably seeded in the intensive care ward and 1 in the nursing ward. Whereas all cases fulfilled given criteria—*i.e.*, they were diagnosed more than 5 days postoperatively and could not be related to any known carriers in the theatre—only 13 could be related epidemiologically to known sources of staphylococci in the intensive-care ward. In another 4 patients, discharge of pus from a superficial wound started more than 10 days after operation. These septic wounds were regarded as infected most probably in the intensive-care ward. Careful assessment, in each case, of clinical data and epidemiological evidence raised doubts as to the validity of the time limit as a means of distinguishing between theatre-acquired and ward-acquired infections. This applied particularly when prophylactic antibiotics were given routinely. Consequently, the place of infection remains in doubt for 5 exogenous infections.

Even if no exact borderline could be drawn between sepsis acquired in the theatre and in the wards, it is obvious that the intensive care ward played a most important epidemiological role in the development of 18 infections (44 per cent). This confirmed our earlier conclusion that human sources, healthy or infected, were critical factors in the intensive-care ward, and frequently initiated vicious circles of cross-infection (Lindbom 1964). To test this conclusion, the interrelation was investigated between patients, their hospital environment and prevalent staphylococci.

It was found that staphylococcal sepsis was the consequence of a complex set of factors, many of which were non-bacterial and unrelated to the intensive-care ward. Some seemed to induce increased susceptibility, *e.g.* extensive surgery, extracorporeal circulation and implantation of foreign bodies. Others provided portals of entry to bacteria, *e.g.* tracheostomy, slow-healing wounds and multiple drainage tubes. Still others, such as operating list and need of prolonged supervision, sometimes created a high-risk postoperative population. Finally, the structure of the unit influenced the risk of sepsis by bringing highly susceptible patients into close proximity, and exposing them to multi-resistant staphylococcal strains prevalent in the *open* intensive-care ward.

To determine the role of the intensive-care ward, standardized environmental studies were made. They showed that staphylococci were

present continuously there but the composition of the flora varied both quantitatively and qualitatively. Frequently, there were fairly abrupt changes which could usually be related to the introduction of a disseminator into the ward. He produced massive contamination not only of his immediate surrounding but of the whole area. Characteristically this patient had septic lesions which could not be covered adequately with dressings e.g. staphylococcal diarrhoea or a septic tracheostomy wound with or without further complications in the form of tracheobronchitis and bronchopneumonia. It was especially noteworthy that—in the absence of cross infection—removal of a disseminator led to the disappearance of his strain from the ward within a couple of days. Two staphylococcal strains in particular seemed to cause infections that predisposed to the status of disseminator. These were staphylococci with clear cut 75/77 patterns or broad reactions of inhibition to group I and group III phages (Thorax I). Clinically they produced a multiform disease that included one or more of the following entities: a septic wound, tracheobronchitis, bronchopneumonia, septicaemia and enterocolitis. Profuse diarrhoea occurred in about one third of the cases; only rarely was it the sole symptom. These strains seemed to have a special affinity for mucous membranes and the ability to induce massive secretion from the respiratory tract and/or diarrhoea which led to massive contamination. Using the technique of Hallander (1962) it could be shown that these strains produced enterotoxin B.

Massive environmental contamination occurred periodically throughout the year. First there was a phase of colonization followed by a second phase of sporadic infections primarily in susceptible patients. Secondary disseminators perpetuated a vicious circle of nosocomial infections. Of patients exposed to disseminators some 20 per cent became transient carriers and 10 per cent persistent nasopharyngeal carriers. Postoperative sepsis developed in 5 per cent. Out of 41 infections 13 were attributed to disseminators of enterotoxin B producing staphylococci. Another 5 infections were classified as auto infections.

Basically these two enterotoxin B producing strains showed a similar pattern of dissemination which contrasted markedly to that of Thorax BP. This state of dissemination was in fact observed only in patient who harboured enterotoxin producing strains in their respiratory tract. The cycle of colonization, infection and dissemination occurred only in tracheostomized patients receiving broad spectrum antibiotics. Thus, in epidemic pattern that was observed in 1961 was repeated several times 1962.

No simple direct relation was apparent between environmental contamination and rate of sepsis. In the spring there was a period of heavy contamination by the 75/77 strain. Although nasopharyngeal colonization was frequent, frank sepsis was rare; if 6 subfebrile cases with profuse sputum are excluded. This situation was reversed in the autumn. Contamination of the environment was not pronounced but sepsis was

fairly frequent. The exact cause of this discrepancy is not known. It was, however, evident that the final outcome was determined by host resistance in the population at risk, degree of exposure and type of prevalent staphylococci. Thus, marked exposure to strains of low infectivity led mainly to sporadic infection of infection-prone patients. When high susceptibility coexisted with marked exposure to a virulent strain the result was frequently an epidemic outbreak of sepsis. In contrast, marked exposure to staphylococci with known epidemic propensities led to a period of sporadic epidemic infection in a population characterized by low to moderate susceptibility. Actually, sepsis seemed to occur in infection-prone patients provided that staphylococci, endogenous or exogenous, could reach susceptible loci in sufficient quantity. If patients were persistent carriers at operation, they frequently contracted endogenous infection, if not, exposure to certain strains commonly resulted in exogenous infection.

SUMMARY

Out of 41 postoperative staphylococcal infections 13 were endogenous and 2/3 exogenous. Human sources—most commonly a persistent carrier or a disseminator—could be established as the ultimate origin of disease-producing staphylococci in 32 infections.

Even if an exact borderline could not always be drawn between sepsis acquired in the theatre and in the wards, the intensive-care ward played a significant role in the development of 18 infections (44 per cent). It was obvious that numerous factors—such as structure of the unit, intramural movements of the patients and rate of turnover—contributed to the creation of a common source reservoir, where characteristics and prevalence of staphylococci influenced the epidemiological pattern. It was nevertheless equally obvious that factors inherent in the patient and his operation were the major determinants whether a given staphylococcus in a given patient was to cause disease.

Sepsis seemed to occur in infection-prone patients, provided staphylococci, endogenous or exogenous, could reach susceptible loci in sufficient quantity. If patients were persistent carriers at operation they frequently contracted endogenous infection, if not, exposure to certain strains commonly resulted in exogenous infection.

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AN ANTIGEN FREE MEDIUM FOR CULTIVATION OF β -HEMOLYTIC STREPTOCOCCI

By

S E HORN and E FALSEN

Received 10 VIII 66

INTRODUCTION

Purification and isolation of antigenically and enzymatically active products from culture filtrates of β hemolytic streptococci have met with difficulties due to the complex protein nature of the media used. Dialyzable media partly eliminated these difficulties and have successfully been employed by, *e g* Dole (1946) Wannamaker (1958) and Halbert (1958). Synthetic media suitable for cultivation of streptococci have also been elaborated (*e g* Zampieri 1950, Ogburn *et al* 1958, Michelson 1964) and would of course from a theoretical point of view be the media of choice for purification of bacterial enzymes and toxins in culture filtrates. However, the yields of bacteria and bacterial products in such media are often poor.

The aim of the present study was to investigate the possibility of using the gel filtration technique for preparing an inexpensive, antigen-free medium containing substances up to a certain molecular weight. The growth promoting effect of the medium and the yield of some bacterial products are reported. The results of some preliminary experiments to separate antigenic streptococcal products from constituents of the medium are also presented.

MATERIAL AND METHODS

Yeast autolysate. Commercial bakers yeast (1 kg) was thoroughly mixed with 1 liter tap water and incubated at 48–50°C for 22 hours. The mixture was heated quickly to 100°C immediately cooled to room temperature neutralized with 5 N NaOH to pH 7.0 and finally centrifuged at 10 000 $\times g$ for 10 minutes. The supernate (designated yeast autolysate) varied in dry weight between 5.7–6.5 per cent (w/v). The recovery was 1.3–1.5 l yeast autolysate per kg yeast. In the gel filtration experi-

We wish to thank *Professor Orjan Ouchterlony* for his criticism and valuable advice during the course of this work. We are also greatly indebted to *Docent Bo*

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ments a twice concentrated yeast autolysate was also employed. This concentration was performed on a Buchi Rotavapor R at 33° C.

Trypticase—yeast autolysate Trypticase, 27 g (BBL 02-148) was dissolved in 100 ml boiling yeast autolysate. After rapid cooling 150 ml twice concentrated yeast autolysate was added and mixed thoroughly.

Buffer solution In the gel filtration experiments as well as in the preparation of different growth media a buffer solution (pH 6.8) of the following composition was used: 0.25 per cent NaHCO_3 , 0.25 per cent KH_2PO_4 , 0.05 per cent $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$.

Supplement In all experiments where growth promoting effect was to be studied, the following substances were added: 2 per cent glucose, 0.03 per cent cysteine HCl, 0.02 per cent L glutamine, 0.0004 per cent Ca pantothenate and 0.2

and eluted with the buffer solution mentioned above. The elution was facilitated

liter

Preparation of dialyzable medium A dialyzable medium was prepared according to the description of Halbert (1958).

Preparation of brain heart infusion (BHI) medium Brain heart infusion, 37 g (Difco) was dissolved in 1 liter distilled water and supplemented with 2 per cent glucose and 0.03 per cent cysteine HCl. Sterilization was performed by autoclaving at 120° C for 20 min.

Analyses

Viscosity was measured at 20° C by means of an Oswald viscosimeter (Kabat & Mayer 1964).

Dry weight estimations were made after heating to 110° C for 12 hours or somewhat longer (up to 20 hours) on viscous samples. Ash was determined after heating at 700° C for 2 hours.

Qualitative amino acid analyses were performed by conventional two-dimensional paper chromatography according to Smith (1953). Cystine was determined as cysteic acid after Schram *et al* (1954). Tryptophan was estimated by the Fischl colorimetric method (1960). Quantitative amino acid analyses were kindly performed by Doc Hallgren at the Sunco laboratories Göteborg Sweden according to the method of Moore *et al* (1958).

DPNase activity was determined according to the technique of Carlsson *et al* (1958).

Streptolysin O (StO) activity was determined according to the technique of Kälébal (1947) and expressed in combining units (Todd units) using a standard anti streptolysin from Statens Seruminstitut Copenhagen.

Streptococcal Strains

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¹ These strains were obtained by courtesy of Dr Rebecca Lancefield, The Rockefeller Institute, New York.

Inoculation Cultivation and Estimation of Bacterial Growth

Inoculations were made by adding 3 per cent (v/v) of a washed streptococcal suspension (optical density 0.51) to the medium to be tested. These streptococci were obtained from a BHI culture incubated at 37° C for 16 hours.

Batch cultivations (in 0.85 liter amounts) were performed under stabilized pH conditions at pH 6.8 by means of an automatic titrator (Radiometer TTT1 Copenhagen) with 5 N NaOH as the neutralizing agent. Tube cultures in triplicate were made when the growth promoting effect of different fractions was tested with strain S 84 and in the tests of the antigen free medium (T1 G 25) with all 22 streptococcal strains. All inoculations were performed at 37° C for 16 hours. Bacterial growth was estimated as optical density (OD) in a Beckman C apparatus. When necessary the cultures were diluted to give an extinction between 0.4 and 0.6. The cultures were homogenized by careful pipetting five times in the tube before the OD was measured. An optical density of 0.51 corresponded to 0.75 mg washed and dried streptococci per ml.

Antisera

Anti CF (CF = culture filtrate) serum was obtained from a sheep which received weekly injections subcutaneously for 8 weeks followed by monthly booster injections for one year. Each immunizing dose consisted of 10 ml ten times concentrated culture filtrate of strain S 84 (grown in BHI medium) suspended in Freund's complete adjuvant. The bleeding used was obtained after the fourth booster injection.

Patient serum was obtained from a 16 year old boy who two weeks earlier had developed clinical and bacteriological signs of an acute streptococcal glomerulonephritis.

Diffusion in Gel Analyses

The double diffusion in gel technique of *Ouchterlony* (1949) was employed in a modified gel chamber technique as described by *Holm* (1965). Media and culture filtrates used in the immunological analyses were concentrated on a Buchi Rota vapor II at 33° C and when necessary diluted with distilled water.

EXPERIMENTS AND RESULTS

Preliminary experiments showed in agreement with *Fox* (1961) that trypsin digested casein was superior to acid hydrolysed casein for supporting growth of β hemolytic streptococci. Still better growth results were obtained when yeast autolysate was incorporated in the medium. *Bernheimer & Pappenheimer* (1942) and *McIlwain* (1946) reported increased growth in the presence of L glutamine and Ca pantothenate which was confirmed by our preliminary findings.

Experiment 1a Sephadex Gel Filtrations and Amino Acid Analyses

In Figs 1 and 2 representative results of gel filtrations of trypsin yeast autolysate on Sephadex G 20 and G 50 columns are shown. The tubes of eluate from the Sephadex G 25 column were combined into 15 fractions each of 250 ml. presence of free amino acids in the fractions was determined by paper chromatography, see Table 1. The first three fractions contained only traces of free amino acids while in fractions 4 and 5 all the amino acids listed in Table 2 (except phenylalanine, tyrosine and tryptophan) were found. In fractions 6 and 7 traces of a large number of free amino acids were present but phenylalanine and tyrosine were predominating and tryptophan completely absent. Tyro

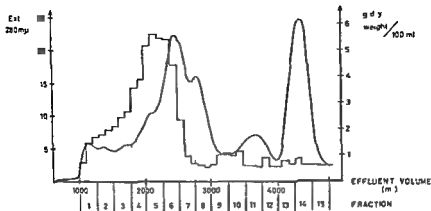


Fig 1

Trypticase-yeast autolysate separated on a 75 × 55 cm Sephadex \square 20 column. Smooth line curve represents the optical density stepped curve the ash content. Fraction numbers are indicated (250 ml/fraction). All eluate in fractions 4-7 and 20 per cent of fractions 8-10 were used in preparing the TYG 20 medium.

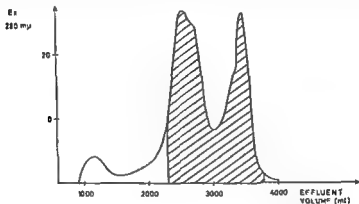


Fig 2

Trypticase-yeast autolysate separated on a 75 × 55 cm Sephadex \square 50 column. All material eluted between 2300 and 3300 ml (shaded) was used in preparing the TYG 50 medium.

sine was the only amino acid found in fractions 8 and 9. No free amino acids could be detected in fractions 10-12, while in fractions 13-15 only tryptophan was recovered. The molecular weight of substances eluted with fraction 1 (the void volume of the column) was above 5000, while the molecular weight of substances eluted with fractions 2 and 3 could be roughly estimated as 2000 to 5000. As may be seen in Fig 1, the ash content of the fractions largely paralleled the optical density, except when amino acids known to have high molecular absorption like tyrosine and tryptophan were present. The fractions

TABLE 2
Growth and Free Amino Acids in Fractions of *Trypticase-Yeast Autolysate*
Separated on Sephadex G-25

Free amino acids*	1	2 3	4	5	Fraction number			8	9	10-11	12-15
					6	7	Traces of phenylalanine and tyrosine, others				
		Trace of some		All except tyrosine, phenylalanine and tryptophan				Tyrosine	Traces of tyrosine	None	Tryptophan
Growth (OD range)	0.08 0.15	0.61 0.72	0.63 0.77	0.77 0.78	0.70- 0.73	0.08- 0.10		0.05- 0.06	0.02 0.08	0 0	0 0

* All amino acids listed in Table 1

from the Sephadex G 25 column were pooled by using all material in fractions 4-7 and 25 per cent of the material in fractions 8-15. The eluates from the Sephadex G 50 column were also pooled, but in this instance all the 1500 ml of the material eluted between 2300 and 3800 ml corresponding to the shaded part of Fig. 2, were combined. No great differences between these two pools were found in dry weight, 2.5 g/100 ml for the Sephadex G 25 pool and 2.1 g/100 ml for the G 50, ash in per cent of dry weight, 28.3 and 26.8, respectively, protein, in per cent of dry weight, 53.6 and 56.8, respectively. Table 2 comprises the results of amino acid analyses of the two pools. The concentrations of free amino acids were lower in the Sephadex G 25 pool than in the Sephadex G 50. The individual amino acids vary quantitatively in both instances. All the amino acids described by Slade *et al.* (1951) as essential for the growth of β hemolytic streptococci were found in both pools.

TABLE 2
Amino Acid Analysis of the Pooled Fractions

Amino acid	Sephadex G 25 pool	Sephadex G 50 pool	Total amino acids (mg/l)
	Free amino acids (mg/l)	Free amino acids (mg/l)	
Aspartic acid*	75	268	1040
Threonine	83	158	410
Serine	74	325	476
Glutamic acid	203	370	2038
Proline	45	98	771
Glycine	63	110	435
Alanine*	226	401	675
Cystine	14	39	90
Valine	190	285	764
Methionine	101	145	275
Isoleucine	168	306	618
Leucine	439	657	1142
Tyrosine	91	232	565
Phenylalanine	268	442	700
Tryptophan	25	17*	372
Lysine	361	596	950
Histidine	47	4	304
Arginine	193	208	494

* Amino acid not essential for growth of β hemolytic streptococci according to Slade *et al.* (1951)

Experiment 1b Growth Promoting Effect of the Fractions and Pooled Material

The growth promoting effect of the fractions collected from the Sephadex G 25 column to which the aforementioned supplement had been added were studied in triplicate cultures using the S 84 strain as a test organism. As may be seen in Table 1 sparse growth or none at all

was obtained in fractions 1 and 7-15, while fractions 2-6 supported good growth. Supplement was added to each of the two pooled fractions (Sephadex G 25, G 50) and the media thus obtained were designated TY G 25 and TY G 50. These two media and three others (BHI medium, dialyzable medium and TY medium) were employed in batch cultures using the S 84 strain as a test organism. The consumption of 5 N NaOH and the optical density of these cultures (grown for 16 hours) were registered and the streptolysin O activity in the culture filtrates determined. Between 3 and 10 batch cultivations were performed in each medium except the dialyzable medium where only one cultivation was made. The results of these experiments are shown in Table 8 where range values with the means in italics are given. It may be seen that the TY, TY G 25 and TY G 50 media promoted a heavy growth of the test strain and that a large quantity of 5 N NaOH was consumed to maintain the pH at a constant level (6.8) during the growth period. The dialyzable medium also promoted heavy growth and the consumption of 5 N NaOH was high. The growth in the BHI medium was good but the highest O.D. value noted was less than the lowest value for the TY, TY G 25 and TY G 50 media. Comparable results were obtained for the consumption of 5 N NaOH.

In Table 3 it may also be seen that the streptolysin O activity varied in the culture filtrates from the different media. The mean activity for the BHI batches was only half the mean levels for the gel filtered media. In some of these batches the culture filtrates were tested for DPNase activity. The activity was found to be as high as 1800-1900 units/ml culture filtrate when the TY G 25 or TY G 50 media were employed as compared to 800-1000 units/ml when the BHI medium was utilized.

TABLE 3

Growth Response and Streptolysin O Production of Strain S 84 Cultivated in Various Media. Mean Values in Italics

Medium	No. of batches	Bacterial growth (in O.D.)	Consumption of 5N NaOH ml/l culture	Streptolysin O activity (units/ml culture filtrate)
Brain heart infusion	10	0.5-0.7 0.9	11 17 29	8-12 16
Dialyzable	1	2.1	31	16
TY	3	2.5-2.6 2.7	35 37 40	16-32 40
TY G 25	10	1.9 2.1 2.4	35 37 39	16 24-27
TY G 50	5	2.2 2.5 2.8	37 40 41	16 24 40

Experiment 2 TY G 25 as a Streptococcal Growth Medium

Ten different strains of β hemolytic streptococci belonging to the Lancefield groups A, C and G were cultivated in the TY G 25 medium

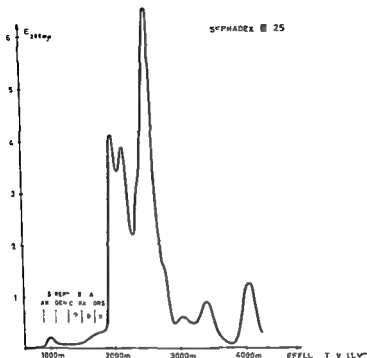


Fig 3

The figure shows the elution curve for a culture filtrate from streptococci grown in TY G 25 medium separated on a 7.5 x 55 cm Sephadex G 25 column. All precipitogenic streptococcal material was eluted with the first small protein peak.

under the aforementioned stabilized pH conditions. The optical density of these cultures varied between 2.1 and 3.9 (mean 2.9). 31–43 ml (mean 38 ml) of 5 N NaOH were consumed per liter culture. For purposes of comparison the same strains were also cultivated in BHI medium under analogous experimental conditions. The bacterial growth in these cultures varied between 0.3 and 1.2 (mean 0.6) and the amount of 5 N NaOH required to maintain the pH at a constant level was 9.5–25 ml (mean 18 ml) per liter culture.

The growth promoting effect of the TY G 25 medium was also tested in tube cultures without pH adjustment using 22 different strains of β hemolytic streptococci belonging to the serologic groups A, C and G. Abundant growth ($OD > 1.0$) was registered in all cultures after 16 hours' incubation at 37° C.

Experiment 3 Purification of Streptococcal Antigens from Cultures Grown in TY G 25 Medium

To investigate the possibility of using gel filtration for isolation of precipitating streptococcal antigens the following experiment was performed. One liter of a culture filtrate from strain S84 grown under

stabilized pH conditions in T₁ G 25 medium was concentrated 20 times in a Buchi Rotavapor. This concentrated material was applied to a 7.5 × 55 cm Sephadex G 20 column. The fractionation was performed as described before but the elution was made with a 0.05 M phosphate buffer (pH 7.0). In Fig. 3 the results of this fractionation experiment are illustrated. The first 800 ml eluted were discarded while the effluent between 800-1800 ml was divided into 50 ml fractions and each concentrated 10 times. The antigenic content of these fractions was compared with that of the 20 times concentrated culture filtrate by means of the double diffusion in-gel method employing anti CF serum and the patient serum. As indicated in the figure the precipitating streptococcal antigens were found in the first small peak eluted from the column well in advance of the major constituents of the medium. The 16 precipitating streptococcal factors demonstrated in the original concentrated culture filtrate were recovered in the early 50 ml fractions. Streptolysin O as well as DPNase activity were also found in the same fractions.

Another liter aliquot of the same culture filtrate was precipitated with ammonium sulfate at 85 per cent saturation at 4° C. The precipitate was dissolved in 10 ml 0.05 M phosphate buffer (pH 7.0) and dialyzed against the same buffer at 4° C for three days final volume 12.5 ml. This highly concentrated (80 times) preparation contained 20 precipitinogens when analyzed with the double diffusion in gel technique against the anti CF serum or the patient serum.

Experiment 4. Antigenicity Testing of T₁ G 25 and T₁ G 50

In order to test the media for possible antigenicity 6 rabbits (two rabbits for each medium) were injected subcutaneously with ten times concentrated T₁. T₁ G 25 and T₁ G 50 media. The immunization schedule employed was the same as that used for the preparation of anti CF in sheep. Each injection consisted of 2 ml concentrated medium in complete Freund's adjuvant. Bleedings were performed after the fourth booster injection and the hyperimmune sera designated anti T₁, anti T₁ G 25 and anti T₁ G 50. These antisera and the anti CF produced by immunization with filtrates from BHI cultures were analyzed with the double diffusion in gel method against the different media.

As indicated in Table 4 five precipitation lines were observed when the T₁ medium was analyzed against anti T₁ serum but no lines were seen when the same medium was tested against the other antisera. When the T₁ G 25 and T₁ G 50 media were tested against the homologous and heterologous immune sera no precipitation lines were observed while the dialyzable medium reacted with anti T₁ and anti CF by forming one line with each of these sera. The BHI medium only reacted with anti CF. At least four distinct precipitates were formed. In comparative analyses it was found that these four lines coalesced

with four of the sixteen lines produced when = 20 times concentrated filtrate from a streptococcal culture grown in BHI medium was analyzed against anti CF

TABLE 4

Number of Immunoprecipitates Noted at Diffusion in Gel Analysis of each Medium with the Various Hyperimmune Sera

Media	Antiserum			
	anti TY	anti TY G 25	anti TY G 50	anti CF
TY	0	0	0	0
TY G 25	0	0	0	0
TY G 50	0	0	0	0
Dialyzable	1	0	0	1
BHI	0	0	0	4

DISCUSSION

One of the first media specifically elaborated for growth of β hemolytic streptococci was presented by Todd & Hewitt as early as 1932. The factors essential for cultivation of β hemolytic streptococci have since been studied by many authors employing partially defined semisynthetic or synthetic media (e.g. McIlwain 1940, Bernheimer & Pappenheimer 1942, Adams & Rose 1945, McIlwain 1946, Slade & Knox 1950, Zampieri et al 1950, Slade et al 1951, Ogburn et al 1958, Fox 1961, Vichelson 1964). From the results of these investigations it could be concluded that a suitable inexpensive base medium for cultivation of streptococci could be produced by employing trypsin digested casein as the essential source of amino acids and peptides and, in addition, yeast extract as the source of purines, pyrimidines and vitamins of the B complex. Furthermore the presence of a variety of trace elements in yeast extracts (Grant & Pramer 1962) may have an additional growth stimulating significance. Various types of supplements have also been employed in streptococcal media to implement the growth promoting effect or the streptolysin O production. The composition of the supplement used in the present study was based on the results reported by Bernheimer & Pappenheimer (1942) and Slade & Knox (1950).

The Sephadex gels are known to separate most substances according to molecular weight and to permit heavy loads (Gelotte 1960, Flodin 1962, Laurent & Killander 1964). Filtration with Sephadex G 25 or G 50 would then represent a convenient way of preparing a growth medium containing substances of a low molecular size and would avoid laborious and time consuming processes like dialysis, salt precipitation or elaboration of synthetic media. The results of the filtration experiments showed that a good separation of high molecular material (eluted with the void volume) from low molecular substances was achieved. Only traces of free amino acids were eluted with the first three fractions

from the Sephadex G-25 column charged with tryptase—yeast autolysate. The analyses of the pooled fractions used in preparations of the TY G-25 and TY G-50 media showed that all amino acids, stated by *Slade et al* (1951) to be essential for cultivation of streptococci, were present in free form. The quantities of these amino acids were small compared to the amounts recommended by these authors but essentially higher than the amounts which, according to *Snell* (1951), allow maximal growth of fastidious lactic acid bacteria. Furthermore, *Slade et al* (1951) discussed the possibility that streptococci can utilize some peptides directly for the synthesis of their own proteins, the large amount of low molecular peptides in the gel-filtered media might compensate for a low concentration of free amino acids.

The results of the cultivation of streptococcal strain S 84 (Group A, type 3) in the TY G-25 and TY G-50 media and the unfiltered TY medium showed that a heavy growth was achieved in all three media. Only minor differences could be noticed concerning consumption of 5 N NaOH, bacterial growth and streptolysin O production. Hence the high molecular weight substances in the TY medium had no specific growth stimulating effect. The brain heart infusion medium, which was used as a commercially available reference medium for streptococci, did not promote as good growth as the gel filtered TY media. This difference was especially pronounced when 10 different streptococcal strains from groups A, C and G were cultivated under identical conditions. In these experiments more than twice as much 5 N NaOH (on the average) was consumed and the bacterial growth was more than three times as dense (on the average) in cultures grown in TY G-25 media compared to cultures grown in BHI medium. That the good growth promoting effect of the TY G-25 medium was not an accidental occurrence for certain streptococcal strains was further verified by tube cultivation employing 22 different strains of β -hemolytic streptococci belonging to the Lancefield's groups A, C and G. All the strains showed an abundant growth ($OD > 1.0$) after 16 hours' cultivation in the TY G-25 medium.

Purification of bacterial antigens in culture filtrates has become a problem of specific interest since it was found that the streptococcal enzyme streptokinase may be utilized in the treatment of medical disease, e.g. thrombosis. In the present investigation a purification experiment was performed by submitting concentrated culture filtrates of streptococci grown in TY G-25 medium to filtration on Sephadex G-25 columns. The streptococcal antigenic factors were eluted with the void volume, i.e. separated from the low molecular constituents of the TY G-25 medium in a single step. It was also shown that ammonium sulfate precipitation (at 85 per cent saturation) was a convenient way to concentrate streptococcal antigens in filtrates from cultures grown in TY G-25 medium. When such a streptococcal antigen preparation was analyzed in double diffusion-in-gel plates against a hyperimmune serum from a sheep (anti CG) or a serum from a patient with streptococcal

glomerulonephritis more than twenty precipitation binds were seen. Similar results have been reported by *Halbert* (1959) and clearly illustrate the multiplicity of antigens in culture filtrates from streptococci.

In the immunological experiments performed to study the antigenicity of the gel filtered media no antigen-antibody reaction could be demonstrated between the media and their homologous antisera. The lack of antigenicity in the TY G 25 and TY G 50 media was further illustrated by analyzing these media against anti-TY sera. Although the TY medium stimulated antibody production in the rabbits these antibodies did not react with the TY G-25 or the TY G 50 media. The dialyzable medium employed was not antigen-free as demonstrated by diffusion-in-gel analyses with both anti-TY and anti CF. Also the BHI medium was found to contain precipitinogenic factors when this medium was analyzed against anti CF serum. One of these factors was common with a factor in the dialyzable medium.

Both the TY G-25 and TY G-50 media seem to fulfil the criteria for an all round streptococcal medium since they are antigen-free, promote good and rapid growth, stimulate the production of extracellular streptococcal antigens and are suitable for isolating high molecular weight streptococcal products from constituents of the medium. It is difficult to choose between these media, but the higher flow rate through Sephadex G 25 compared to Sephadex G 50 dictated the use of TY G-25 in the present study. These results are largely in accord with preliminary results on this topic reported by *Falsen & Holm* (1964).

SUMMARY

The preparation of streptococcal media containing elements of low molecular size by gel filtration of trypticase-yeast autolysate through Sephadex G-25 and G 50 is described. Amino acid analyses of the media showed that all amino acids known to be essential for the growth of β -hemolytic streptococci were present in a free form. Heavy growth and high streptolysin O activity were produced by β hemolytic streptococci in these media and at higher levels than in a commercially available streptococcal medium. Immunological experiments showed that the gel filtered media lacked antigenic properties and were suitable for the preparation of extracellular streptococcal antigenic materials freed from medium constituents.

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AN INTRACELLULAR INHIBITOR OF STREPTOCOCCAL DPNase IN BETA-HEMOLYTIC STREPTOCOCCI

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Received 10 VIII 66

Since *Carlson et al* (1957) first described a streptococcal DPNase¹ acting on the nicotinamide-ribose linkage of DPN the enzyme has been studied in different aspects (*Bernheimer et al* 1957, *Lazarides & Bernheimer* 1957, *Kellner et al* 1958, *Bernheimer* 1960, *Petersen* 1961, *Petersen et al* 1961 *Petersen* 1962a, 1962b, *Holm & Kajser* 1965). Its role in streptococcal infections is not clear. *Bernheimer et al* (1957) suggested an association between DPNase production and leucotoxic activity. DPNase has also been claimed to influence the development of streptococcal nephritis (*Bernheimer* 1960). *Kellner et al* (1958) and *Petersen* (1962) observed raised DPNase antibody levels after streptococcal infection in man.

The aim of the present investigation was to study the production of DPNase by streptococci and the protection of the microorganism against the enzyme. The DPNase activity in extra and intracellular preparations from streptococcal strains belonging to Lancefield's groups A, C and G was investigated. The final results of a preliminary report (*Holm & Kajser* 1965) on the characteristics of an intracellular DPNase inhibitor in certain streptococcal strains are given.

MATERIAL AND METHODS

Streptococcal strains. Ten beta hemolytic streptococcal strains² from Lancefield's groups A, C and G were investigated. The strains used are listed in Table I and the Lancefield designations are given.

Preparation of extracellular material (EC). The streptococci were grown in brain heart infusion broth (Difco 37 g/l) supplemented with sterile filtered cysteine HCl

¹ DPN (Disphosphopyridine nucleotide) is nowadays often called NAD (Nicotinamidadeninedinucleotide).

² These strains were kindly supplied by *Dr Rebecca Lancefield*, The Rockefeller Institute, New York.

We wish to express our deep gratitude to *Prof. Dr. R. Bernheimer* for his valuable criticism. We are also indebted to *Dr. Eng. Eneid Falsen*, Mrs. *Elisabet Wale* and *Eng. Eneid Falsen*.

The skilful technical assistance of *Miss Ulla Wikström* is very much appreciated.

and glucose at final concentrations of 0.03 per cent and 2 per cent respectively. In parallel experiments the gel filtered antigen free medium described by Falsen & Holm (1964) was employed. The cultivations were performed under stabilized pH conditions at 6.2 and 6.8 using an automatic titrator and employing 5 N NaOH as the neutralizing agent. Estimation of growth was performed by measuring the optical density as described in a preceding paper (Falsen & Holm 1964). After sixteen hours of cultivation at 37°C the streptococcal cultures were centrifuged at 1500 $\times g$ for half an hour at 4°C. The supernatant was filtered through a Göttingen No. 6 membrane at 4°C and kept in the cold room. This material was designated extra cellular material (EC).

Preparation of intercellular material (IC) The wet bacterial sediment from each culture was washed three times with saline and finally suspended in 4 volumes of buffered saline pH 7.0 (M/15 phosphate buffer). The suspension was homogenized in a Raytheon 200 watt 10 kc oscillator for half an hour and thereafter freeze-pressed five times in the press described by Fdebo (1960). After thawing the pressed material was mixed with glass beads and shaken at 4°C for three hours and finally centrifuged at 12000 $\times g$ at 4°C for half an hour. The clear slightly yellow supernate was designated IC (intercellular material).

Anti EC serum was obtained from a sheep which had been immunized by repeated subcutaneous injections of EC from strain S 84 (Group A Type 3) using Freund's adjuvant technique. The immunization schedule implied weekly injections of 10 ml ten times concentrated culture filtrate during 8 weeks followed by one monthly injection of the same amount for one year. Bleedings were performed one week after the last injection.

Anti IC + FC serum was obtained from a sheep immunized in a similar way as described above by injections of mixtures of IC and EC of several strains of group A streptococci.

Anti IC sera were produced with Freund's adjuvant technique in rabbits by six weekly injections of IC from one DPase negative (Ti 1197) two DPase positive group A strains (S 84 and B 737 34 41) and from one moderately DPase positive group G strain (F 63 A). The corresponding immune sera were designated anti IC A1, anti IC A3, anti IC A49 and anti IC G.

Anti IC A3 + IC A1 Equal amounts of anti IC A3 and IC A1 were incubated at 37°C for 30 min and stored overnight at 4°C. The supernatant after centrifugation at 1500 $\times g$ for 20 min was decanted and designated anti IC A3 + IC A1.

Trypsin treatment of IC One ml trypsin solution (1 mg/ml M/15 phosphate buffer pH 8.0) was added per ml IC and the mixture was incubated for 30 minutes at 37°C. Trypsin inactivation was achieved by adding 1 ml of a buffered saline solution pH 8.0 of soy bean trypsin inhibitor (1 mg/ml ABC England). The final DPase inhibitory capacity was tested by means of a preparation with known DPase activity (300 units/ml). The effect of the various reactants were tested as shown in Table 2.

Gel filtration of IC IC material was fractionated on Sephadex G 75 columns (25 \times 82 cm) using a 0.05 M phosphate buffer pH 7.0 in 0.5 M NaCl. The fractions were collected by means of a LKB fraction collector using a 5 ml siphon and concentrated in visking tubing (hebo T 106) against polyethylene glycol 6000. As reference substances with known molecular weight recrystallized preparations of lysozyme and/or pepsin (ABC England) were filtered through the same Sephadex column.

Dialysis of IC Dialysis was performed in visking tubing (hebo T 106) with a pore diameter of 50 Å.

Ultrafiltration of IC Ultrafiltrations were performed on the LKB model 6300 A ultra filter at 4°C. According to the manufacturer's specification the pore diameter corresponds to a molecular size of about 30000.

DPase determination was made according to the technique of Carlson *et al* (1957). This is a slight modification of the method described by Kaplan *et al* (1951): one unit being the amount of enzyme that destroys 0.01 μ M DPase in 7.5 minutes at 37°C. In our tests the DPase concentration after addition of NaCN in excess corresponded to an extinction of 0.6 in a Beckman DU spectrophotometer (340 m μ 1 cm cuvettes). The decrease in optical density was linearly related to DPase activity only in a limited region (ext 0.45-0.20).

DP\ase inhibition test In these tests we have chosen a DP\ase concentration corresponding an extinction of 0.25. Thus higher extinction values correspond to the presence of DP\ase inactivating substances.

IC preparations (0.05 ml) and the same amount of the DP\ase solution (in 0.1 M phosphate buffer pH 7.3) were mixed. After 2 min. at room temperature 0.4 ml of the DP\ase solution mentioned above was added and the mixture was incubated for 75 min. at 37° C. The enzymatic action was stopped by cooling in ice water and the addition of 3 ml 5% NaCl. The optical density was measured. Suitable blanks and controls were run in parallel. DP\ase inactivation by anti DP\ase and inhibitor neutralizing effect of anti inhibitor sera were analysed in a similar way in all sera used.

The quantitation of DP\ase inhibition was based on the DP\ase unit—one inhibiting unit being the amount that inhibits one unit of DP\ase.

Immunological analyses Comparative double diffusion in gel analyses were performed according to the method of *Ouchterlony* (1958) in a modification described by *Holm* (1965). In the immunoelectrophoretic analyses the technique described by *Wadsworth & Hanson* (1960) was used, an elaboration of the original method of *Grabar & Williams* (1953). Adequate separations were obtained on 9 × 12 cm glass plates with a field strength of 7 V per cm applied for 80 minutes. The antigen basins were placed near the cathode as most of the precipitinogens in IC move towards the anode during the electrophoresis. Photographic registration of the precipitation patterns was performed according to the description of *Wadsworth* (1963).

TABLE 1

Intracellular DP\ase Inhibitor Effect Compared with Intra- and Extracellular DP\ase Activity in Various Strains of Streptococci

Group	Beta streptococcus Type	Strain*	DP\ase activity in FC†	DP\ase activity in IC‡	DP\ase inhibition in IC‡
A	1	T1 119 7	—	—	—
A	3	S84	+++	—	+++
A	3	C 203S	+++	—	+++
A	4	T4 95 Rbo	+++	—	++
A	49	B737 34 41	+++	—	+++
A	50	B514-33 3	+++	—	++
C		h61 0 13	—	—	—
C	20	T20J (Neil)	++	—	++
G	16	T16 J	++	—	++
G		F68 A	++	—	+

—
+
++
+++

† EC = Extracellular material

‡ IC = Intracellular material

* The strain numbers refer to the designation made by R. Lancefield

RESULTS

DP\ase Activity in Extra- and Intracellular Materials

As may be seen in Table 1 DP\ase activity was found in FC from 5 of the 11 group A strains tested. DP\ase activity was not demonstrated in the sixth strain (T1 119 7). The highest DP\ase activity (1800 units/ml) was detected in cultures from the two Type 3 strains when cultivated in the antigenic medium. One of the two group C

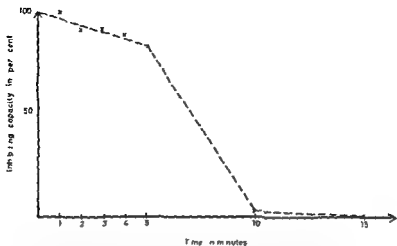


Fig 1

Figure showing the inactivation of the intracellular DPNase inhibitor in relation to time at 60° C. The inhibitor power at different intervals is expressed as per cent of the initial inhibitory capacity of intracellular material

strains produced moderate amounts of DPNase while the other was DPNase negative. The two group G strains tested showed moderate DPNase activity. No significant differences in DPNase production were noted if the cultivations of the different strains were performed at pH 6.8 or 6.2.

In no experiment was active DPNase found in IC from any of the strains tested.

DPNase Inhibition by Intracellular Materials

Table 1 also shows the inhibitory capacity of IC from streptococcal strains belonging to groups A, C and G and the DPNase activity in the corresponding culture filtrates. The highest inhibitory capacity was found in IC from the two Type 3 strains which also showed the greatest extracellular DPNase activity. Furthermore it was found that the inhibitor was active not only against DPNase from the corresponding strain or type but also against DPNase produced by streptococci belonging to other serological groups. As may be seen only DPNase producing strains showed DPNase inhibiting effect in IC.

Characterization of the DPNase Inhibitor

Thermostability. Storage for 6 months at 4° C did not inactivate the DPNase inhibitor in IC. Nor did heat treatment for 45 min. at 37° C lower the inhibitory effect. However, at 60° C a complete inactivation of the DPNase inhibitor was obtained in 15 min. (Fig. 1).

Heat treatment of EC for 10 min. at 60° C completely destroyed the DPNase activity.

Trypsin stability The results of trypsin treatment of the inhibitor are shown in Table 2. As may be seen the DPNase inhibitor was destroyed by trypsin. It was also shown that neither DPNase nor the inhibitor were affected by the soy bean trypsin inhibitor used to inactivate the trypsin.

TABLE 2
Effect of Trypsin on DPNase Inhibitor

DPNase inhibitor	Trypsin solution	Proportion of reactants Soy bean trypsin inhibitor solution	Buffer solution	DPNase units added	Final DPNase activity (in units)
0	0	0	3	800	800
1	0	0	2	800	< 100
1	1	1	0	800	800
1	0	1	1	800	< 100
0	1	1	1	800	800
0	0	1	2	800	800

Dialysis and ultrafiltration Dialysis of IC in visking tubing with a pore diameter of 50 Å against buffered saline showed that the inhibitor was not dialysable. Ultrafiltration experiments showed, however, that the inhibitor appeared to be at the borderline of penetrability through the ultrafilter permitting passage of substances of a molecular size up to 30 000.

Gel filtration Fractionation experiments of IC were performed on Sephadex G 75 columns. In Fig 2 a fractionation experiment of IC A3 (S 84) is illustrated. High DPNase inhibitory activity was found in

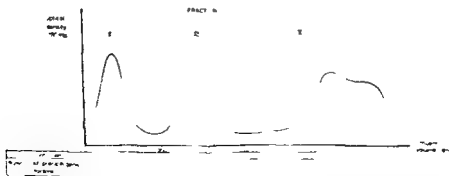


Fig 2

The curve represents the separation of intracellular material (S 84) on a Sephadex G 75 column. The DPNase inhibitory activity was almost exclusively found in fraction III while most of the other intracellular precipitinogenic factors were eluted in fractions I and II.

fraction III Fractionation of IC from five other DPNase positive strains showed almost identical results. No inhibitory effect could be detected in any fraction of separated IC from DPNase negative strains. In order to evaluate the molecular size of the inhibitor, reference substances with a molecular weight of 17,500 and 34,500 (lysozyme and pepsin respectively) were filtered through the same Sephadex column. These two substances were eluted on either side of the protein peak of IC fraction III which indicated that the molecular size of the inhibitor could be roughly estimated at 20,000–30,000.

Antigenicity Sera from rabbits immunized with IC preparations from DPNase positive and negative strains were analysed for the presence of inhibitor neutralizing antibodies. The results of the analyses of two anti-sera are summarized in Table 3. As may be seen the DPNase inhibitory effect of IC A3 (S84) was neutralized by the corresponding antiserum. This antiserum also neutralized the inhibitory effect of IC from the other type 3 strain tested. It is further evident from the table that the inhibitor neutralizing antibodies were not type or group specific as the antibodies in anti-IC A3 could also neutralize the inhibitory effect in IC from streptococci belonging to other group A strains as well as to groups C and G. Anti-IC A3 (S84) did not inactivate DPNase in IC from any of the DPNase positive strains tested. Antiserum produced with a DPNase negative strain (anti-IC A1) did not neutralize the inhibitory effect of IC preparations from any DPNase positive strain, nor were DPNase antibodies detected in this serum.

TABLE 3

Inhibitor Neutralizing Effect of Anti IC A3 and Anti IC A1 on IC Preparations from certain Streptococcal Strains

Group	Beta streptococcus		Inhibitor neutralizing effect of	
	Type	Strain	Anti IC A3	Anti IC A1
A	3	S84	+	—
A	3	C203S	+	—
A	4	T4 95 Rb5	+	—
A	49	B737 34 41	+	—
A	50	B514 33 3	+	—
G	20	F20J (Well)	+	—
G	10	T16J	+	—

The immunoelectrophoretic pattern obtained when IC A3 (S84) was separated and analysed against anti-IC + FC serum is illustrated in Fig 3. At least twenty separate precipitation lines were revealed with the crude IC, but when Sephadex gel fraction III of IC A3 (S84) was tested against anti-IC + FC serum only two precipitation lines were found. These lines were designated II and xλ. Only the xλ immunoprecipitation band was observed when fraction III of IC A1 (lacking DPNase inhibiting effect) was analysed in the same way against anti IC + FC and

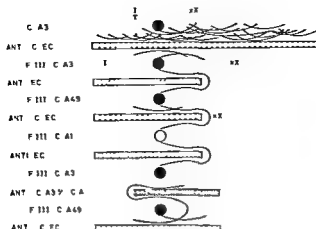


Fig 3

Schematic drawing of immunoelectrophoretic separations of streptococcal intracellular material or fractions thereof developed with different anti streptococcal sera. *Filled circular basins* DP\ase inhibitory materials. *Open circular basin* Material lacking DP\ase inhibitor. *Dotted long basins* Immune serum containing DP\ase inhibitor neutralizing antibodies. *Shaded long basins* Immune serum lacking DP\ase inhibitor. The inhibitor-anti inhibitor immune precipitate is designated II.

anti EC serum. This precipitation line (x) was also the only one seen when fraction III of IC A3 (S84) was analysed against anti EC serum which lacked inhibitor neutralizing antibodies.

The precipitation pattern in the lower part of the figure illustrates the antigenic relationship between the i factors in sephadex gel fractions III of IC from two different inhibitory positive group A streptococcal strains analysed against anti IC A3 (S84) serum which had previously been absorbed with IC A1. Only one precipitinogen was revealed in each fraction and the coalescence of the lines indicated the immunological identity of the i antigen in the two fractions. Serological reactions of identity shows that the x antigen was common to IC and EC of DP\ase positive and negative strains. Similar results were obtained using the comparative double diffusion technique of Ouchterlony.

DISCUSSION

Since DP\ is an importance coenzyme in cellular respiration it is likely that the bacterial cell producing DP\ase must also have the capacity to control the activity of the latter. Such a control mechanism has been described by Berthelmer *et al.* (1951) who observed that streptococcal RNA inhibits the action of streptococcal DP\ase.

In our studies most human pathogenic streptococcal strains belonging to Lancefield's group A, C and G were capable of producing DP\ase which was detected in IC. The only DP\ase negative streptococci

fraction III Fractionation of IC from five other DPNase positive strains showed almost identical results. No inhibitory effect could be detected in any fraction of separated IC from DPNase negative strains. In order to evaluate the molecular size of the inhibitor, reference substances with a molecular weight of 17,500 and 34,500 (lysozyme and pepsin respectively) were filtered through the same Sephadex column. These two substances were eluted on either side of the protein peak of IC fraction III which indicated that the molecular size of the inhibitor could be roughly estimated at 20,000-30,000.

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TABLE 3

Inhibitor Neutralizing Effect of Anti IC A3 and Anti IC A1 on IC Preparations from certain Streptococcal Strains

Group	Beta streptococcus		Inhibitor neutralizing effect of	
	Type	Strain	Anti IC A3	Anti IC A1
A	3	S84	+	—
A	3	C203S	+	—
A	4	T4 95 Rb5	+	—
A	49	B737 34 41	+	—
A	80	B514 33.3	+	—
G	20	T20J (Neil)	+	—
G	16	T16J	+	—

The immunoelectrophoretic pattern obtained when IC A3 (S84) was separated and analysed against anti-IC+EC serum is illustrated in Fig. 3. At least twenty separate precipitation lines were revealed with the crude IC but when Sephadex gel fraction III of IC A3 (S84) was tested against anti-IC+EC serum only two precipitation lines were found. These lines were designated α I and α X. Only the α X immunoprecipitation band was observed when fraction III of IC A1 (lacking DPNase inhibiting effect) was analysed in the same way against anti-IC+EC and

that an intracellular inhibitor may be liberated during long term cultivation resulting in depressed DPNase activity

Schwarz *et al.* (1956) have described a DPNase inhibitor and its corresponding enzyme produced by *Proteus vulgaris* and *Bacillus subtilis*. However, these DPNase attack the pyrophosphate group of DPN and not the linkage between nicotinamide and ribose as the streptococcal DPNase does

Kern (1956) detected a DPNase and a corresponding inhibitor in *Mycobacterium butyricum*. This enzyme cleaves the nicotinamide—ribose linkage of DPN just as the streptococcal DPNase does. In bacterial extracts he found no DPNase activity because of the presence of an active inhibitor. By boiling he could selectively destroy the inhibitor and thus produce an enzymatically active intracellular extract. In the present study intracellular materials from DPNase positive streptococci were also found to lack DPNase activity. However, heat treatment could not be used to selectively inactivate the inhibitor because streptococcal DPNase was as heat sensitive as the inhibitor

SUMMARY

Intracellular preparations from DPNase positive streptococcal strains were found to contain an antigenically active DPNase inhibitor. The results of immunological analyses showed that the inhibitor was neither type nor group specific. A protein nature was suggested by the results of heat and trypsin treatment. The molecular weight could be roughly estimated at 20000–30000.

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RESPIRATORY VIRUS DISEASE IN INFANCY AND CHILDHOOD IN COPENHAGEN 1963-65

An Estimation of the Aetiology Based on Complement Fixation Tests

By

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A substantial part of the respiratory infections in infancy and childhood can now be associated with several agents which are known to be responsible for different proportions of the various respiratory disease syndromes. Even though the complement fixation test has been shown to be of comparatively low sensitivity in these age groups (3, 20, 42) it is still the most convenient test for use in epidemiological surveys. Reports from recent years (15, 31, 44) have clearly established that the respiratory syncytial (RS) virus and the parainfluenza viruses are more important as aetiological agents for both upper and lower respiratory tract disease in childhood than the influenza viruses, while the adeno viruses take up a position intermediate between these.

Paired sera from infants and children admitted to hospitals in Copenhagen over the years 1963, 1964 and 1965 mainly for respiratory tract disease have been examined for complement fixing antibodies against influenza, parainfluenza, adeno and respiratory syncytial viruses. It has been attempted to establish the frequencies of infections caused by the viruses mentioned, which can be diagnosed by means of complement fixation tests. It has been shown that virus isolation is a more sensitive method for detecting infection than the complement fixation test in parainfluenza, adeno and respiratory syncytial virus infections in infancy (3, 30, 42, 4, 29). The moderate sensitivity of the complement fixation tests employed was taken into consideration when the results presented in this paper were evaluated.

MATERIAL AND METHODS

The material consisted of paired sera collected from paediatric departments in hospitals in Copenhagen during the periods 1963 to May 1965. Only sera from the age groups 0-9 years have been examined.

This investigation was supported by the J. J. Petersen foundation.

The author is indebted to Dr A. P. N. Magnus MD, Statens Serum Institut, Copenhagen for permission to publish results of tests for complement fixing antibodies to adenovirus and influenza virus performed in the influenza department at Statens Serum Institut.

In the Influenza department at Statens Seruminstitut 473 paired sera have been tested for complement fixing antibodies against influenza A H C and adeno virus. Of these 473 sera we have tested 404 for antibodies against the RS virus 398 for antibodies against parainfluenza virus type I 329 for antibodies against parainfluenza type II and 368 for antibodies against parainfluenza virus type III. Furthermore 272 of the above mentioned 473 sera have been tested for complement fixing antibodies against ornithosis.

Antigens employed: 1) RS virus complement fixing antigen prepared in Hep 2 cell cultures as described previously (18). 2) Parainfluenza I viral antigen (C-F) Burroughs Wellcome & Co. London. 3) Parainfluenza II (CA) C-F antigen Microbiological Associates Bethesda USA. 4) Parainfluenza III (HA 1) C-F antigen Microbiological Associates Bethesda USA. 5) Ornithosis phosphatide antigen prepared as described previously (45). 6) Mumps antigen Behringswerke Marburg Germany. 7) Influenza A B and C complement fixing antigens were prepared in the Influenza department in embryonated eggs employing the following virus strains: A I R8 B Lee and C Am 1233. 8) Adenovirus complement fixing antigen was prepared in the influenza department from HeLa cell cultures infected with the adeno virus type 4.

Complement fixation tests employed: 1) For RS virus antigen as earlier described (17). 2) For all the parainfluenza virus antigens and with the ornithosis antigen and the mumps antigen mentioned above the technique of Bradstreet & Taylor (1) has been used. 3) In the Influenza department at Statens Seruminstitut the technique of Fulton & Dumbell (12) as modified by Svedmyr et al. (37) was employed in tests for influenza and adeno virus complement fixing antibodies.

Control antigens and both positive and negative control sera were included in all tests series.

Intervals between the collection of the paired sera were from 5 to 18 days with a mean of 10 days. All sera were heated for 30 minutes at 56° C and stored at -20° C until examined.

Abbreviations used: 1) C-F = Complement Fixation. 2) P I = Parainfluenza type I. 3) P II = Parainfluenza type II. 4) P III = Parainfluenza type III. 5) RS = Respiratory Syncytial. 6) Infl C = Influenza type C.

EXPERIMENTAL

Occurrence of Antibodies against Parainfluenza Type I Complement Fixing Antigen

Fig. 1 shows the distribution according to age of 308 paired sera examined with this antigen. It can be seen that the majority of sera comes from patients below the age of three years. Fig. 1 also shows the age distribution of sera containing antibodies (dilution 1:8 being the lowest dilution examined) and of sera showing more than a two fold increase in titre. The percentage of sera showing presence of antibodies rises from 11 per cent in the age group 0 years to 63 per cent in the age group 4 years. There is only a slight increase in the percentage of sera showing presence of antibodies in the age groups above 4 years. This fact is illustrated in Fig. 4. It can be seen from Fig. 1 that there is no accumulation of sera showing more than a two fold increase of titre in any particular age group.

Occurrence of Antibodies against Parainfluenza Type II Complement Fixing Antigen

Fig. 2 presents the distribution according to age of 329 paired sera examined with this antigen and of sera containing antibodies (indicated

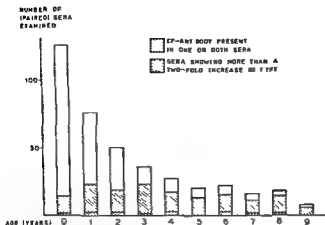


Fig 1

Distribution according to age of 398 paired sera from patients admitted to hospitals in Copenhagen during the period September 1963 to May 1965

All sera were examined for G-F antibodies to parainfluenza virus type 1
Those showing presence of antibody are indicated

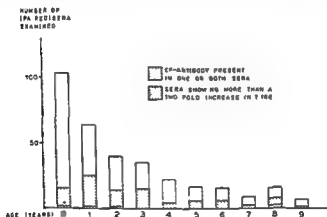


Fig 2

Distribution of 100 paired sera from patients admitted to hospitals in Copenhagen during the period September 1963 to May 1965

All sera were examined for G-F antibodies to parainfluenza virus type 1

Those showing presence of antibody are indicated

in the same way as in Fig 1) The percentage of sera showing presence of antibodies (dilution 1:8 being the lowest dilution examined) rises from 15 per cent at age 0 to 3 years. In the older age groups the bodies show a decreasing trend, being due to the small number of sera examined in the older age groups

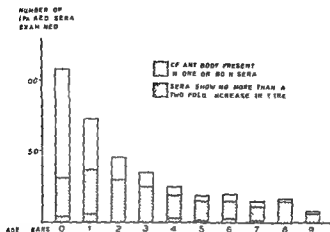


Fig 3

Distribution according to age of 368 paired sera from patients admitted to hospitals in Copenhagen during the period September 1963 to May 1965

All sera were examined for C-F antibodies to parainfluenza virus type III
Those showing presence of antibody are indicated

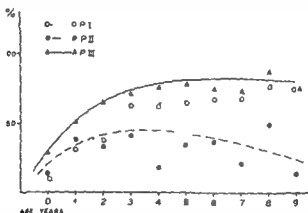


Fig 4

Distribution according to age of percentages of paired sera containing C-F antibodies to parainfluenza viruses type I, II and III from patients admitted to hospitals in Copenhagen during the period September 1963 to May 1965

Because of the difference in numbers of sera examined in the different age groups it is not possible to say whether there is accumulation of sera showing more than two fold increase of titre in any age group

Occurrence of Antibodies against Parainfluenza Type III Complement Fixing Antigen

Fig 3 shows the distribution according to age of 368 paired sera examined and of sera found to contain antibodies against this antigen (serum dilution 1/8 being the lowest dilution examined). The per-

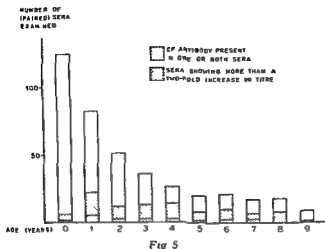


Fig 5

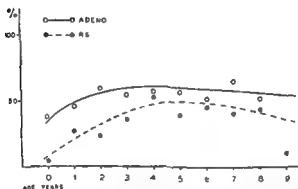


Fig 6

Distribution according to age of percentages of paired sera containing C-F antibodies to adeno and RS viruses from patients admitted to hospitals in Copenhagen during the period September 1962 to May 1963

centage of sera containing antibodies rises from 29 per cent in patients below 1 year to approximately 80 per cent in the age groups above 5 years. There is no definite accumulation of paired sera showing more than a two-fold increase of titre in any age group examined with this antigen.

In Fig 4 the percentage of sera containing antibodies against the three parainfluenza C-F antigens mentioned above in the age groups 0-9 years are shown. It can be seen that the percentage of sera containing antibodies against type III antigen increases more steeply and reaches a higher level than the percentages of sera containing antibodies

against type I and II antigens Fig 4 demonstrates clearly that the type II infection has been the rarest parainfluenza infection encountered in Copenhagen in recent years

Occurrence of Antibodies against the RS Virus Complement-Fixing Antigen

Fig 5 shows the distribution according to age of 404 paired sera examined and of sera containing antibodies against this antigen. The percentage of sera containing antibodies increases from approximately 5 per cent in the age group 0 years to approximately 50 per cent in the age group 4 years. This is also shown in Fig 6. In the age groups above 4 years the percentage of sera containing antibodies shows a tendency to decline. As was the case with the percentages of sera showing antibodies against parainfluenza type II antigen, this decrease in the older age groups may also be fallacious where this antigen is concerned, because of the comparatively small number of sera examined in these age groups. No definite difference in the frequencies of paired sera showing more than a two fold increase of titre could be found for the age groups examined, as is apparent from Fig 5.

Results of Tests for Influenza A, B, C and Adeno Virus Complement Fixing Antibodies

At the Influenza department 473 paired sera have been examined for these antibodies as described above. No increase of titre was found against influenza A and only one increase of titre, in a patient with a lower respiratory tract infection, against influenza B. Six patients showed an antibody response to influenza C and 15 patients showed an antibody response to adeno virus C-F antigen. The percentages of paired sera showing presence of antibodies against the last mentioned antigen in one or both sera (dilution 1:4 being the lowest serum dilution examined) are shown for the age groups examined (0-11 years) in Fig 6. It can be seen that there was an increase from approximately 35 per cent in the age group below 1 year to approximately 60 per cent for the age groups above 3 years.

Results of Tests with Mumps and Ornithosis Complement-Fixing Antigens

272 paired sera were also tested for C-F antibodies against ornithosis but no presence of antibodies was found. Because of the known antigenic relationship between parainfluenza viruses and mumps virus, 23 of the 31 paired sera which showed antibody responses to parainfluenza virus antigens were also tested with mumps antigen, but no concomitant increases of titre were found.

TAB
*Distribution According to Main Diagnosis of Posit
 and Examined for Different Vir*

Diagnosis	RSV		P I		Patients examin	
	N exam *	Pos †	N exam	Pos	N exa	
Pneumonitis	196	13 (6.6)§	193	7 (3.6)	16	
Bronchopneumonitis	26	2 (7.7)	25	1 (4.0)	2	
Bronchiolitis	1	0 -	1	0 -		
Acute Bronchitis	38	2 (5.3)	40	0 -	3	
Acute Laryngitis	11	0 -	12	0 -		
Acute Rhinopharyngitis	10	0 -	10	0 -		
Catarrhalia	15	0 -	15	0 -	1	
Acute Tonsillitis	11	1 (9.1)	9	0 -		
Acute Supp Otitis Media	12	0 -	10	0 -		
Febrile Convulsion	18	0 -	15	0 -	1	
Others	66	0	88	3 (4.4)	5	
Total	404	18 (4.5)	398	11 (2.8)	32	

* N exam = Number examined

† Numbers in brackets indicate percentage of paired sera examined from patients with

against one of the mentioned antigens are represented by the hatched squares. These also assist in the evaluation of which season the different respiratory infections might show the highest prevalence. As can be seen from Fig. 7, there is some accumulation of RS infections (top graph) and of adeno infections (bottom graph) in the winter months. As it is evident from these graphs that a larger number of sera have been examined in the winter months, these accumulations might only be apparent. No seasonal accumulation of parainfluenza or influenza C infections could be found. This is evident from Fig. 7, which also demonstrates higher frequencies of parainfluenza I and III infections than of parainfluenza II infections.

Distribution of Sera Examined and of Increases of Titre Obtained According to Diagnosis

The examined sera were divided according to the different diagnoses shown in the column to the left in Table 1. These diagnoses represent the main diagnoses attached to the patients at the time of discharge from the hospitals. "Others" comprise various neurological, haematological and urinary tract disorders, but both in this group and in the group listed as "febrile convulsion" the major part of the patients had a respiratory infection as a secondary diagnosis. In the group listed as acute laryngitis a few of the patients had the diagnosis acute laryngo-tracheobronchitis. Of the 473 patients examined for influenza C or adeno virus infections only 41 did not have a respiratory tract infection. That is 91 per cent of the patients examined had either an upper or a

*Sera from Children Hospitalized in Copenhagen 1963-65
Infections by Complement Fixation Test*

or (infection)

I	P III		Infl II		Adeno	
	Pos	\ exam	Pos	\ exam	Pos	\ exam
3 (1.8)	178	7 (3.9)	238	3 (1.3)	238	11 (4.6)
1 (4.5)	25	1 (4.0)	26	2 (7.7)	26	0 -
0 -	1	0 -	1	0	1	0 -
0 -	36	1 (2.3)	41	0	41	0 -
0 -	12	2 (16.7)	17	0 -	17	1 (5.9)
0 -	10	0 -	11	0	11	1 (9.1)
0 -	13	0 -	11	0	16	1 (6.3)
1 (12.5)	9	0	11	0 -	11	1 (9.1)
0 -	7	0 -	11	0 -	12	0 -
0 -	13	0 -	11	0	19	0 -
1 (1.7)	64	3 (4.7)	81	1 (1.2)	81	0 -
6 (1.8)	368	14 (3.9)	473	6 (1.2)	473	10 (3.2)

† Pos = Number of positives (\geq four fold increase of titre)
the diagnosis in question

lower respiratory tract infection. It is evident from Table 1 that the majority of patients examined had a lower respiratory tract infection.

Of the 70 paired sera showing an increase of titre to one of the antigens listed in Table 1, only four showed a double increase to two of the antigens. Three showed a double increase to parainfluenza type I and type III antigen and are listed as type I infections in Table 1. The fourth showed a double increase to parainfluenza type II and type III and is listed as a type II infection in Table 1.

Table 1 demonstrates that no major difference in percentages of positive sera in the different categories of diagnoses listed could be found. Comparison of these percentages is furthermore made difficult by the differences in the number of sera examined. A number of sera in the group listed as "others" showed increase of titre to the parainfluenza antigens or to the influenza C antigen. With exception of the increase seen to the influenza C antigen all other increases of titre in this group appeared in patients who had a respiratory tract infection as a subdiagnosis.

In Table 2 all sera from patients with a respiratory tract infection as main diagnosis have been divided according to the principal localization of the infection: upper or lower. The percentages of positive sera (indicated in the brackets) obtained with the different antigens employed only showed a difference between upper and lower respiratory tract infection when the RS antigen was used. The results suggest that the RS virus infection was mainly associated with lower respiratory tract infection in the children examined.

TABLE 2

Distribution According to Localisation of Respiratory Disease of Paired Sera from Children Hospitalised in Copenhagen 1963-65 with Respiratory Infections
The Number of Sera Examined and Number of Sera Giving a Positive Reaction in Complement Fixation with the listed Antigens Are Indicated

Examined for	Upper respiratory infections			Lower respiratory infections		
	N exam	Pos †	%	N exam	Pos	%
RS	86	1	1.2	279	17	6.1
P I	85	1	1.2	277	10	3.6
P II	61	2	3.3	234	4	1.7
P III	79	3	3.8	256	11	4.3
Infl C	102	0		330	5	1.5
Adeno	102	4	3.9	330	11	3.3

* N exam = number examined

† Pos = Positives \geq four fold increase of titre

TABLE 3

Distribution According to Age of Paired Sera from Children Hospitalised in Copenhagen 1963-65 with Pneumonitis of Bronchopneumonitis Examined by Complement Fixation Test with the Various Respiratory Virus Antigens Listed

Examined for	Number of positive sera (> 4 fold increase of titre)					%
	Age groups (years)					
	0	1	2-4	5-9	10-14	
RSV	2/78*	4/54	6/71	5/51	17/254	6.7
Infl C	0/103	3/67	2/79	0/54	5/303	1.7
P I	1/79	2/49	5/80	1/49	9/257	3.5
P II	1/65	1/42	1/71	3/42	6/220	2.7
P III	2/68	5/47	2/78	2/46	11/239	4.6
Adeno	7/103	2/67	2/79	1/54	12/303	4.0

* The numbers to the right of the oblique line indicate number of sera examined with the virus antigen in question in the age group concerned

It can be seen from Table 1 that a major part of the sera examined were from patients with diagnoses of pneumonitis or bronchopneumonitis. Also some sera in the groups named "febrile convulsion" or "others" in this Table originated from patients with a subdiagnosis of pneumonitis or bronchopneumonitis. In Table 3 all sera from patients with these two diagnoses are divided according to age of the patients. The number of sera examined and the number of positive sera found in each group with the six antigens in question are presented in this table. Some slight accumulation of positive sera in 1-4 year old patients examined with influenza C or parainfluenza I antigen and in patients below one year of age examined with adeno virus antigen can be seen. Somewhat different numbers of paired sera were examined with the different antigens. When this fact is kept in mind it is estimated that approximately a quarter of children with pneumonitis or

bronchopneumonitis were infected with either RS virus influenza virus type C, parainfluenza virus or adeno virus as determined by complement fixation tests. These viruses were associated with approximately 6-7 per cent, 1.5-2 per cent, 11-12 per cent, and 3-4 per cent of these infections respectively.

DISCUSSION

Evidence of parainfluenza infection demonstrated either by recovery of the viruses or serologically has established the endemic nature of these viruses (5, 30). A survey showing the percentages of complement fixing antibodies to these viruses in different age groups of children should therefore give an estimate of the prevalence of parainfluenza infection in the different age groups, together with the relative importance of the different main types of these viruses. As shown in this paper the percentages of children who possess antibodies to these viruses increase sharply during the first four to five years of life. The same increase of percentages of children who possess antibodies have been demonstrated by other authors who either tested for the presence of neutralizing antibodies (10, 30) or for the presence of haemagglutination (HI) antibodies (23, 35). The percentages of neutralizing antibodies reported in the different age groups (10, 30) are very similar to analogous percentages for complement fixing antibodies reported in this paper. The finding that neutralizing antibodies to parainfluenza III appear at an earlier age and are more frequently encountered in all age groups examined than neutralizing antibodies to parainfluenza I and II (10, 30), is also confirmed when complement fixing antibodies are tested for, as shown in the present report.

The distribution according to age of the percentages of sera showing presence of complement fixing antibodies to the RS virus given in this paper is almost identical to a similar distribution reported by *Vivell & Deibel* (43) but the percentages are lower than those in a similar distribution of antibodies to this virus reported by *Hambling* (14). The distribution according to age given in this paper shows somewhat higher percentages of sera containing antibodies in the age groups concerned (0-9 years) than those which have been reported previously by the author (17). On the other hand in this previous report (17) the sera forming the basis for the age distribution of antibodies were collected from the whole of Denmark. Furthermore it was pointed out (17) that the percentages of sera containing antibodies in the age groups concerned (0-9 years) might vary in successive years.

An incidence of sera containing antibodies to adeno virus complement fixing antigen increasing from 3 per cent in the age group 0 year to approximately 60 per cent in the age group 11 years has been reported in this paper. This incidence corresponds closely to similar incidences given by *Potter & Shedden* in England (32) and by *Jordan*

in America (22) and confirms the high prevalence of these infections in infancy and early childhood

The epidemic pattern of influenza A and B and RS virus infections as opposed to the more endemic nature of adeno and parainfluenza virus infections is now well established (5, 16, 34, 36), even though small circumscribed epidemics have often been encountered with the two last mentioned kinds of viruses. The low incidence of parainfluenza infections during all seasons found by other authors (30) has been confirmed by the findings described in this paper. All three types of parainfluenza infections were encountered during all seasons in Copenhagen without any accumulations. Severe lower respiratory tract infections in children caused by adeno viruses or RS virus are encountered most often during the winter and spring (6, 16, 40). In our serological survey we have encountered RS virus infections and adeno virus infections most often during the months October to March. As we have tested most sera in these months this finding might be fallacious, as mentioned above.

Several serological studies have supported the great importance of the RS virus as aetiological agent for severe respiratory disease in infancy and early childhood (9, 13, 25, 28, 29). Parrott *et al* (29) found most increases of titre by complement fixation in infants 7-12 months of age in a large group of patients with severe respiratory disease, but could find no association between RS infections and any particular age group in patients with mild respiratory disease. We found most RS infections in patients with lower respiratory tract disease, but could not find any association with a particular age group. The proportion of cases of respiratory disease which were found to be associated with RS infection in the present survey was lower than found by others (25, 29) but this fact may be explained by a number of circumstances. RS virus is a very important agent in the bronchiolitis syndrome (9, 13, 29), which was only diagnosed in one patient in our survey. This could of course be a question of difference in the clinical terminology employed. More important is the fact that there was only an interval of approximately 10 days between the blood samples in the majority of pairs of sera examined. According to Ross *et al* (33) results of complement fixation tests with RS virus antigens were improved by postponing the collection of the convalescent serum until the fourth or fifth week after the start of illness. On the other hand we did titrate our RS antigen against a pool of sera from small children as recommended by these authors (33). Even when sufficient consideration is given to technical problems the complement-fixation for RS virus is still probably somewhat inefficient in early infancy (4) and a large part of the patients in the present survey belonged to this age group. This moderate sensitivity of the complement-fixation test for the diagnosis of RS virus infection in early infancy and in childhood (20-30 per cent and 80-90 per cent respectively) (4, 29) must be taken into consideration when

the results obtained are evaluated. Therefore it is estimated that RS virus have been responsible for approximately 10-11 per cent of the pneumonitis and bronchopneumonitis infections investigated and not only 5-7 per cent as listed in Table 3.

The percentage of respiratory disease in children in Copenhagen found to be associated with parainfluenza infection as diagnosed by complement fixation 9-10 per cent (Table 2) is similar to results obtained by this method in Germany (44), in Holland (3), and in America (15). About 70-80 per cent of infants and children infected with parainfluenza viruses have been reported to show homotypic and/or heterotypic antibody responses by complement fixation (30). Therefore it is estimated that approximately 14-15 per cent (and not 10-11 per cent as listed in Table 3) of the patients with pneumonitis or bronchopneumonitis have been infected with parainfluenza viruses. We were unable to confirm the close association between parainfluenza type I infection and the croup syndrome found by other laboratories (2, 26) but the number of patients with this syndrome in our survey was rather small. According to Parrott (31) parainfluenza type II plays a larger part in the aetiology of bronchopneumonitis, bronchiolitis and acute bronchitis in children than does parainfluenza type I. We were unable to confirm this even though only three sera gave a double increase of titre with parainfluenza I and III antigens and were listed as parainfluenza I infections as mentioned above.

The close antigenic relationship between the different parainfluenza viruses makes a specific aetiological diagnosis by complement fixation with these antigens uncertain (3-30). In the present survey an increase of titre to P III antigen was seen in one (17 per cent) of the demonstrated increases of titre to P II antigen and in three (27 per cent) of the demonstrated increases of titre to P I antigen. Even so these antigens supplement one another in the establishment of the (unspecific) diagnosis of parainfluenza infection by serological means. Proper consideration must also be given to the antigenic relation of the parainfluenza viruses to the mumps virus in the evaluation of the results of a serological survey with the former viruses (9) even though this relationship is closer in sera from adults than from children (24). No double increases of titre to parainfluenza and mumps antigen were found in this survey, as mentioned above.

It has been estimated that adenoviruses are responsible for a few per cent of respiratory diseases in civilian populations (22-41). This is in accordance with the results presented in this survey. Varpaska *et al.* (42) have reported that 19 per cent of 223 infants and children from whom adenoviruses were recovered showed a complement fixing antibody response. These authors found that the highest percentage of antibody responses occurred in infants below the age of 12 months and that antibody responses occurred almost exclusively in infants and children who were ill. In this survey most of the increases

of antibody were also seen in the age group below 12 months. It was reported (42) that 39 per cent of the patients who were ill and from whom adenoviruses had been recovered from a throat swab specimen showed an antibody response. In this paper 3-4 per cent of patients with upper or lower respiratory infections showed an antibody response to adeno virus complement-fixing antigen (Table 2). If the complement-fixation employed in this report have been of similar sensitivity as the test employed by the authors referred to above (42) then it can be estimated that a least 6-8 per cent of infants and children hospitalized in Copenhagen 1963-65 with respiratory infections have had an adeno virus infection. Several reports from recent years (7, 11, 20, 21, 38) have established the adeno viruses as important aetiological agents in severe lower respiratory tract infections especially in cases of pneumonitis in infancy and childhood. *Jansson & Wager* (19) examined 517 patients (all ages) with pneumonitis or bronchopneumonitis for complement-fixing antibodies to adeno type 2 antigen. Of these 517 patients 5 per cent showed a significant antibody response and half of the higher antibody titres were seen in the age group 1-5 years. In the present study there was no accumulation of adeno virus antibody responses in lower respiratory infections and no association between antibody response and any particular disease syndrome could be demonstrated.

Influenza C virus is usually considered to be responsible only for cases of mild upper respiratory tract disease (27). Occasionally this virus may be associated with more severe respiratory disease in children. *Wenner et al* (46) found influenza C to be an associated cause in four of 42 infants with either bronchiolitis or pneumonitis. In the present survey antibody responses to influenza C were found in five of 264 cases of pneumonitis or bronchopneumonitis, all of which were in the age group 1-4 years.

SUMMARY

Infants and children aged 0-9 years, admitted to hospitals in Copenhagen 1963-65 mainly for respiratory tract illnesses have been examined for complement fixing antibodies to influenza A, B, C, parainfluenza I, II, III, adeno and respiratory syncytial viruses. The majority of the patients were also tested for complement-fixing antibodies to ornithosis.

Parainfluenza III infections were found to be more prevalent than parainfluenza I and II infections. RS and adeno virus infections were mainly diagnosed during the months October to March while no seasonal variations were observed for the parainfluenza infections. A majority of the diagnosed RS infections were seen in patients with lower respiratory tract infections, while no preference of localization was found for adeno, parainfluenza or influenza infections. A majority of the sera examined came from patients with diagnoses of pneumonitis.

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MYCOPLASMA PNEUMONIA INFECTION IN INFANTS AND CHILDREN IN COPENHAGEN 1963-65

Incidence of Complement-Fixing Antibodies in Age Groups 0-9 Years

By

ALLAN HORNØSLETH

Received 29 VII 66

Several reports have established the importance of *Mycoplasma pneumoniae* (Eaton agent) as aetiological agent in acute respiratory tract disease in military and adult civilian population groups (2, 6, 10, 11, 12, 17, 24, 26), but few have been concerned with the possible importance of this agent in respiratory tract disease in childhood (2, 5, 17, 21). Complement fixation with dissimilar antigens was employed by Thomas *et al* (25) in the study of cases of primary atypical pneumonia (PAP). Liu *et al* (22) described the development of specific antibodies to the Eaton agent in PAP patients, they employed the fluorescent antibody technique. The growth of the Eaton agent on artificial media was accomplished by Chanock *et al* (7) who also described the method for preparation of complement fixing antigen (8). Other methods for preparation of this antigen have been given by Jansson (15) and by Kenny (18).

Complement-fixing antibody responses to *Mycoplasma pneumoniae* (*Mpn*) have been determined in infants and children admitted to hospitals in Copenhagen over a 20 month period. The percentages of children showing presence of antibodies and the incidences of increases of titre for the age groups 0-9 years have been established.

MATERIAL AND METHODS

The material consists of 367 paired sera collected from paediatric departments in hospitals in Copenhagen during the period September 1963 to May 1965. These sera form part of a number of sera which have been examined for complement fixing antibodies against different respiratory viruses as described elsewhere (14). Approximately 90 per cent of the sera originated from patients whose main diagnosis was a respiratory tract infection.

The technique of Bradstreet & Taylor (3) was employed in all complement fixation tests. All the specimens were heated for 30 minutes at 56° C and stored at -20° C until examined. Control antigen and both positive and negative sera were included as controls in all test series.

The author is indebted to Dr A. Lind, the Department of Toxoplasmosis and Viral Diseases, Statens Serum Institut, Copenhagen for carrying out tests for antibodies against *Mycoplasma pneumoniae* by the indirect fluorescent antibody technique.

Antigen employed *H pa* complement fixing (C-F) antigen made by Robbin Laboratories Inc., Chapel Hill N.C., U.S.A. The antigen was titrated against a positive serum with a fluorescent antibody titre of 1750 kindly supplied by Dr A. Lind Statens Seruminstitut.

The fluorescent antibody tests (FAT) performed at the department of Toxoplasmosis and Viral diseases Statens Seruminstitut have been described (21). Cold agglutinin titrations were also carried out in this department as described previously (20).

The methods employed in C-I tests with Influenza parainfluenza adeno and respiratory syncytial viruses have been described elsewhere (14).

EXPERIMENTAL

Distribution According to Age of C-F Antibodies against Mycoplasma pneumoniae

Table 1 shows the distribution according to the patients' age of the 367 paired sera examined. It can be seen that antibody was present in the majority of sera from patients over the age of 11 months. Even though the sera examined constitute a very selected material infection with *H pa* must have been very prevalent in children in Copenhagen during the last few years. The percentages of sera with a four fold increase of titre (the column to the right in Table 1) show a gradual increase with age but the small numbers of sera examined in older age groups do not allow any definite conclusion to be drawn in this respect.

TABLE 1

Distribution according to patients' age of 367 paired sera examined for C-F Antibodies to Mycoplasma pneumoniae

Age group	No. tested	Antibody titre ≥ 8 in one or both sera		≥ 4 fold increase in titre	
		No.	per cent	No.	per cent
0-5 months	41	4	9.8		
6-11 "	80	33	41.5	3	3.8
1 year	67	47	70.1	1	1.5
2 years	46	35	76.1	1	2.2
3 "	34	22	64.7	2	5.9
4 "	29	14	48.3		
5 "	20	14	70.0	3	15.0
6 "	21	19	90.5	2	9.5
7 "	14	10	71.4	2	14.3
8 "	17	15	88.2	2	11.8
9 "	5	4	80.0	1	20.0

Distribution According to Diagnosis of C-F Antibodies to Mycoplasma pneumoniae

This distribution is shown in Table 2. The diagnoses listed to the left in this table represent the main diagnosis attached to the patients at the time of discharge from the hospitals. Others comprising various neurological, haematological and urinary tract disorders 37 on 6.

sera examined in the group listed as "others" and 14 of the 15 sera examined in the group listed as "febrile convulsion" came from patients with a respiratory tract infection as a secondary diagnosis. That is, 92.1 per cent of the 367 patients examined had a respiratory tract infection. From Table 2 can be seen that five of the 17 patients who showed a significant antibody response belonged to the group listed as "others". Of these five two had a pneumonitis and two had an upper respiratory tract infection as a secondary diagnosis, whilst the fifth had a main diagnosis of anaemia, but no respiratory tract infection. That is 14 (82 per cent) of the 17 significant antibody responses came from patients with a lower respiratory tract infection, but it must be stressed that these patients contributed the majority of sera examined, as is evident from Table 2. Of 338 patients with respiratory tract infections 16 or 4.7 per cent showed a significant antibody response to the *M. pn* C-F antigen employed. Table 2 reveals furthermore that a large part of the sera which showed increasing, decreasing or high non-shifting antibody titres came from patients with clinical diagnoses of viral pneumonitis or primary atypical pneumonitis.

TABLE 2

Distribution According to Main Diagnosis of 367 Paired Sera from Children Hospitalized in Copenhagen 1963-65 Examined for Antibody Responses to Mycoplasma pneumoniae by C-F Tests

Diagnosis	Number of sera examined	≥ 4 fold increase in titre	≥ 4 fold decrease in titre	Antibody titre* ≥ 512
Bronchopneumonitis	23	1 (4.3)†	0	1 (4.3)
Pneumonitis	112	3 (2.7)	0	2 (1.8)
Viral pneumonitis	27	1 (3.7)	2 (7.4)	4 (14.8)
Primary atypical pneumonitis	38	6 (15.8)	1 (2.6)	3 (7.9)
Acute bronchitis	36	1 (2.8)	0	0
Bronchiolitis	1	0	0	0
Acute laryngitis	9	0	0	0
Acute rhinopharyngitis	8	0	0	0
Catharrhalis	14	0	0	0
Acute tonsillitis	9	0	0	0
Febrile convulsion	15	0	0	0
Acute suppurative otitis media	10	0	0	0
Others	65	5 (7.8)	0	0
Total	367	17 (4.6)	3 (0.8)	10 (2.7)

* No increase or decrease in titre

† Numbers in brackets indicate percentage of sera with the diagnosis in question

Relationship between C-F Antibody Response to Mycoplasma pneumoniae and Corresponding Cold Agglutinins

313 of the 367 paired sera examined for C-F antibodies were also examined for cold agglutinins (CA). According to the test employed for determination of CA (20) a titre ≥ 64 or a four-fold increase of

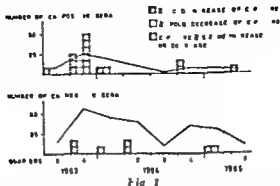


Fig. 1

titre was considered positive. Of the 313 sera examined 239 were found to be CA negative whilst the remainder (75 sera) were found to be CA positive. In Fig. 1 is shown the distribution according to season of these CA negative and CA positive sera. Each of the paired sera showing an increase or a decrease of titre together with sera which gave a titre ≥ 512 without decrease or decrease of titre in C-I tests are indicated by a little square. Most of the sera which were found by C-I tests to be associated with a *W. pn* infection were CA positive. In the fourth quarter of 1963 11 out of 25 CA positive sera (44.0 per cent) were associated with a *W. pn* infection but only 2 out of 36 CA negative sera (5.6 per cent) were associated with this infection as found by C-I tests. The variation according to season of the number of sera examined makes it difficult to say whether there was any seasonal variation in the number of sera which showed an increase or decrease of titre.

With the object of obtaining an estimation of how the C-I titres varied in comparison with the corresponding CA titres during the first few weeks following the start of illness, an analysis of 22 paired sera with high antibody titres, increase or decrease of antibody titres to *W. pn* and with known CA titres was carried out. According to the time of blood sampling the 44 *W. pn* C-I titres obtained together with the corresponding CA titres were divided up into groups each of which represented a period of one week. In this way five to 13 titres were included in each of four groups corresponding to the first four weeks following start of illness. The mean titres were calculated for each group and in Fig. 2 these weekly mean C-I and corresponding CA titres are shown in logarithmic form. Both the C-I titres and the CA titres seem to reach a maximum in the third week following start of illness but the CA titres seem to decline later in the following weeks.

Relationship between Corresponding C-I and Fluorescent Antibody Responses

Table II shows the titres obtained in 14 paired sera examined for these two kinds of antibodies. With the fluorescent antibody test (FAT) em

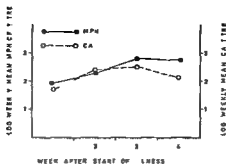


Fig 2

ployed (21) a four-fold increase of titre or a titre of ≥ 160 was considered positive. It can be seen in Table 3 that there was a close correlation between the two kinds of antibodies. The six pairs of sera which showed either high antibody titres or gave a four-fold or greater increase of titre by C-F test all gave a positive FAT. The pairs of sera which showed either low or negative antibody titres by C-F test all gave a negative FAT.

TABLE 3

Antibody Responses in 14 Paired Sera Examined for Antibodies to Mycoplasma pneumoniae by Fluorescent Antibody Test (FAT) and by Complement Fixation Test (C-F)

Serum	FAT	C-F
14657 I	40 (80)	16
- II	160 (320)	256
14840 I	≥ 40	64
II	320	>1024
14879 I	≥ 40	16
II	320 (640)	512
15518 I	80	32
- II	80 (160)	128
16634 I	40 (80)	8
II	160	64
16698 I	320	1024
- II	640	512
14816 I	≥ 40	32
II	≥ 40	32
15919 I	<40	32
- II	<40	16
16020 I	<40	32
II	<40	8
16653 I	<40	<8
- II	<40	8
16867 I	<40	<8
- II	<40	8
17035 I	<40	<8
II	<40	8
17033 I	<40	<8
II	<40	<8
16802 I	<40	<8
II	<40	<8

Double Infections with Mycoplasma pneumoniae and Respiratory Viruses

All 367 paired sera examined for C-F antibodies against *M. pn* have been examined for C-F antibodies against influenza, adeno and respiratory syncytial viruses (14) and the majority of these sera have also been examined for C-F antibodies against parainfluenza viruses (14).

Of the 30 paired sera which responded to *M. pn* C-F antigen with an increase or decrease of titre or revealed a titre ≥ 512 as listed in Table 2, 24 have been examined for C-F antibodies against all the different respiratory viruses mentioned above. Nine sera with *M. pn* C-F titres ≥ 512 and two sera which showed decreases of titre against *M. pn* did not show any double infection against the viruses mentioned. Of 13 sera which showed an increase of titre against *M. pn* one showed an increase of titre to respiratory syncytial virus, one showed an increase of titre against influenza type B and four showed increases of titre against parainfluenza viruses, (one against type I, one against type III and two against both type II and type III). That is, 6 of 13 sera (46.2 per cent) with increases of antibody against *M. pn* also showed double infections with respiratory viruses.

The patient with a double infection of *M. pn* and influenza type B was a three year old girl with a diagnosis of pyuria, but with a subdiagnosis of recurrent rhinopharyngitis. Double infection with *M. pn* and respiratory syncytial virus was seen in a seven year old boy with bronchopneumonitis. Four girls with ages ranging from 9 months to 8 years had double infections with *M. pn* and parainfluenza viruses. 1) the patients aged nine months had bilateral pneumonitis. 2) a patient aged six years had been admitted with convulsions and a rash and was under observation for epilepsy, 3) one patient aged eight years had a diagnosis of pyuria and a subdiagnosis of cathartitis and 4) another patient aged eight years had the diagnosis of infectious mononucleosis and a subdiagnosis of primary atypical pneumonia.

DISCUSSION

Several reports support the value and specificity of C-F tests in the study of PPLO infections. Card (4) employed an antigen made from a PPLO strain which was isolated from a genital infection when she examined different categories of patients and healthy population groups for C-F antibodies. She found the highest incidence of antibodies in patients who were attending venereal disease clinics. Lemelle (19) studied the immunological responses in rats and mice which were inoculated with several different strains of PPLO. She found a high degree of correlation between the titre of serum C-F antibody and the presence of PPLO in the tissues of these animals. She furthermore observed that the quantity of antibody increased with increasing dissemination of the infection in the animal (19). Chanock *et al* (8)

compared the C-F test with the FAT in the diagnosis of Eaton agent infection in marine recruits and found the C-F test to be approximately 80 per cent as sensitive as the FAT. Both Goodburn *et al* (11) and Biberfeld *et al* (2) found good correlation between the C-F test and the FAT in the diagnosis of *M pn* infections. Grayston *et al* (12) examined 463 patients (all ages) with respiratory tract infections for infection with *M pn*. When the C-F test was employed in 40 of 57 patients from whom this organism was isolated an increase of titre was demonstrated in all patients who had a titre < 16 in the first blood sample. The specificity of the C-F test employed in the present report is supported by the good correlation which was found between C-F titres and fluorescent antibody titres in the sera examined (Table 3). Furthermore the CA positive group of patients included most of the infections which could be associated with *M pn* by C-F test (Fig. 1). This finding is in accordance with observations made by Jansson *et al* (17) and by Grayston *et al* (12).

The percentages of sera in different age groups showing presence of C-F antibodies to *M pn* given in this paper are higher than analogous percentages given by Grayston *et al* (12). Even though a smaller number of sera were examined by these authors in the age groups concerned, 0-9 years, the wide fluctuation in the prevalence of *M pn* (13) is a more probable explanation of this discrepancy.

M pn infections have been diagnosed most often in older children and in adults when either isolation of the infectious agent or demonstration of increases of antibody titres have been employed in patients with respiratory tract illness (2, 12, 17, 21). Jansson *et al* (17) found the highest incidence of positive antibody responses by C-F tests in the age groups 6-30 years. Grayston *et al* (12) found the highest isolation rate of *M pn* in patients aged 10-19 years, but found the C-F test to be a more sensitive diagnostic procedure than isolation of the agent. Grayston *et al* (12) isolated 4 strains of *M pn* from 60 patients aged 0-9 years. Biberfeld *et al* (2) diagnosed by C-F test and FAT 5 cases of *M pn* infection in a group of 13 patients aged 6-10 years, but no cases in a group of 14 patients aged 0-5 years. Lind (21) isolated 10 strains of *M pn* from 19 patients aged 5-14 years old but none from 27 patients aged 0-4 years. In the present survey 2.4 per cent of patients aged 0-4 years and 13.0 per cent of patients aged 5-9 years showed antibody responses to *M pn*, but the difference in number of sera examined in these two age groups, 290 and 77 respectively, makes an assessment of these percentages difficult. Chanock *et al* (5) found an increase of antibody by FAT in 10 per cent of 152 infants and children with lower respiratory tract illnesses.

Chanock *et al* (6) found that 40 per cent of a group of recruits with respiratory illness who were found to be infected with *M pn* by FAT were CA positive. Of 37 cases of respiratory illness (mainly pneumonitis) found to give positive antibody responses in C-F test and FAT by

Biberfeld *et al* (2) 17 were C-I positive. When 246 patients with pneumonia all ages were examined for C-I antibodies against *M pn* (Jansson & Wager 1964) approximately two thirds of the patients who gave a positive antibody response were found to be C-I positive. Grayston *et al* (12) reported that approximately 75 per cent of a group of patients (all ages) with respiratory tract illnesses that gave a positive antibody response by C-I were C-I positive whilst only 25 per cent of the C-I negative were C-I positive. Grayston *et al* (12) also observed that a positive C-I response was a more frequent finding among children who showed increase of antibody by C-I test than among adults who gave a positive antibody response by C-I. As described in this paper the majority of patients who gave a positive antibody response by C-I test were C-I positive.

Finland *et al* (9) described that maximum titres of cold agglutinins were seen between the 11th and 24th day following the start of illness (primary atypical pneumonia) and that titres decreased in the third or fourth week. Similar observations concerning the cold agglutinin titres were made by Jansson & Wager (16). Grayston *et al* (12) found that maximum C-I titres were reached in the beginning of the fourth week following start of illness and that the titres only decreased moderately in the following few months. This temporal distribution of the C-I titres obtained seems to follow the pattern for fluorescent antibody titres as described by Lim *et al* (22). According to the results presented in this paper maximum C-I titres seem to be reached mainly in the third week following start of illness.

Lim (21) found evidence of double infection with *M pn* and adenoviruses in two out of 63 patients with pneumonia. Baernstein *et al* (1) found increases of C-I antibody against the respiratory syncytial virus in two out of six children from whom *M pn* was isolated. Some double infections with *M pn* and adenoviruses or influenza virus type A have been observed by Marmion & Hers (23). In the present paper concomitant infections with *M pn* and respiratory viruses were frequently observed. Six out of 13 patients who showed increases of titre against *M pn* were also infected with a respiratory virus. These double infections were most frequently seen with parainfluenza viruses but did not occur in any particular age group.

SUMMARY

367 paired sera from infants and children aged 0-9 years admitted to hospitals in Copenhagen during the period 15 September 1963 to May 1965 have been examined for C-I antibodies against *Mycoplasma pneumoniae*. The majority of sera from patients over the age of 12 months showed presence of antibody. The majority of patients with respiratory tract infections showed significant antibody responses. The percentage of patients who showed antibody responses was higher in the age

group 5-9 years than in the age group 0-4 years. The major part of patients who could be associated with a *M. pneumoniae* infection by C-F tests were found among the group who showed presence of cold agglutinins (CA). Maximum C-F and CA titres were generally reached in the third week following the start of illness. Of 13 paired sera which showed an increase of C-F titre six showed double infections with respiratory viruses.

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activity was tested by filling the enzyme into 10 mm diameter holes cut in the agar and reading after 24 hours incubation at 37° C.

Spectrophotometric determinations Infrared absorption spectra were recorded using a Perkin Elmer spectrophotometer model 237. Samples for infrared analysis were prepared either as dry film on Irtan 2 cells or pressed into tablets with KBr in vacuo under a pressure of 10 tons per sq. inch as recommended by Perkin Elmer. Similar absorption bands were obtained by either method although the samples as KBr tablets gave a better resolution. Extinction measurements on the fractions

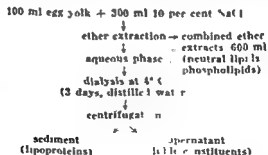
EXPERIMENTAL

It was demonstrated earlier (5) that the egg yolk reaction as studied by the agar plate method was composed of three phenomena, namely a clearing, precipitation, and the formation of fatty material on the agar surface. Repeated filling of the enzyme in the holes of egg yolk agar plates and incubation at 37° C. overnight revealed a number of rings of clearing and precipitation.

Egg yolk is a complex mixture of lipids, lipoproteins and other constituents, and the egg yolk reaction can be caused by more than one enzyme attacking different substrates in egg yolk. Egg yolk was therefore fractionated in order to identify the substrate(s) which were involved.

Identification of the Substrate(s) in Egg Yolk

The method described by Chargaff (1) for the preparation of lipoproteins was used for the fractionation of egg yolk. Egg yolk was mixed with ten per cent sodium chloride, as outlined in the following scheme, and the free lipids were extracted with diethylether three times and after removal of the ether, the aqueous phase was dialysed to precipitate the lipoproteins which could be collected by centrifugation.



The precipitated lipoproteins were dissolved in 10 per cent sodium chloride solution and precipitated again by dialysis and then taken up in 50 ml of sodium chloride solution. Addition of a few drops of ether dissolved the turbidity to give an opaque solution.

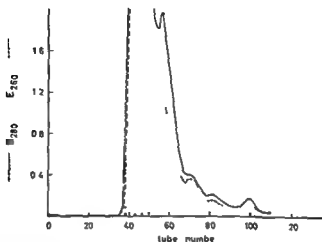


Fig. 1

Separation of egg yolk lipoproteins on Sephadex G 200 (Dotted region indicates the location of the turbid phase)

The different fractions derived from egg yolk were tested for substrate activity. When mixed with the agar and poured into Petri plates the ether extract and the lipoproteins gave a turbidity whereas the agar plates containing the soluble constituents were clear. No reaction was obtained with the enzyme on plates containing the ether extract and the water-soluble fraction. The lipoprotein fraction gave reactions similar to that obtained with unfractionated egg yolk, yielding the clearing and precipitation phenomena as well as appearance of fatty material on the surface. Thus the substrate was located in the lipoprotein fraction which was further fractionated by gel filtration using Sephadex G 200 μ el (Pharmacia Uppsala).

Chromatography of lipoproteins on Sephadex G 200 5 ml of the lipoprotein solution was chromatographed on Sephadex G 200 with a bed size of 1.5 cm diameter and 120 cm length using 0.1M Tris HCl buffer of pH 8.0 and containing 1M sodium chloride. The effluent was collected in 1 ml lots in an automatic fraction collector. Extinction measurements at 260 and 280 m μ on the fractions gave the distribution pattern indicated in Fig. 1. The first fractions were turbid and the turbid material tended to rise to the surface on standing. Centrifugation at 3,000 \times collected the turbid material as a sheet on the surface leaving a clear yellow bottom phase.

The different fractions were tested for substrate activity. The contents of tubes up to number 65 when mixed with the agar gave a turbid suspension. The clear yellow phase obtained on centrifugation also gave a turbidity on mixing with agar. It was found that the turbid substance which was collected on the surface was the active fraction whereas the clear yellow solution was devoid of activity. The turbid substance dissolved on the addition of a few drops of ether. These properties of the

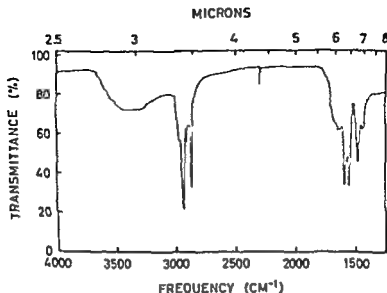


Fig. 2

Infrared absorption spectrum of the fatty material from egg yolk agar plates (KBr pellet)

turbid substance corresponded to the description given in literature (2) for lipovitellin and the clear yellow solution apparently contained lipovitellin. The substrate active in the egg yolk reaction was thus identified as lipovitellin.

Identification of the Products of the Egg Yolk Reaction

The egg yolk reaction was composed of clearing and precipitating reactions with the liberation of fat appearing over the precipitation region on the agar surface. The precipitate and the fat which apparently represented the end products of the egg yolk reaction could be easily removed with the aid of a nickel spatula and suspended in acetone. No such material could be removed from the agar surface which had not reacted with the enzyme.

Infrared absorption spectrum and melting point. The fatty material and the precipitate collected from several egg yolk agar plates after reaction with the enzyme were isolated and examined for absorption in the infrared region. The infrared spectrum of the material (Fig. 2) showed the presence of a weak bonded —OH ($2.9\text{--}3.25\ \mu$), R—C—H_2 ($3.42\ \mu$), R—C—H ($3.5\ \mu$) and C=C deformations in the region of $6\text{--}7\ \mu$ possibly with a C=C stretching weak absorption at $6.605\ \mu$. The absorption was not characteristic of any one substance but seemed rather to be a mixture. Microscopic examination showed that it consisted of an oily substance and an amorphous material, the latter melt

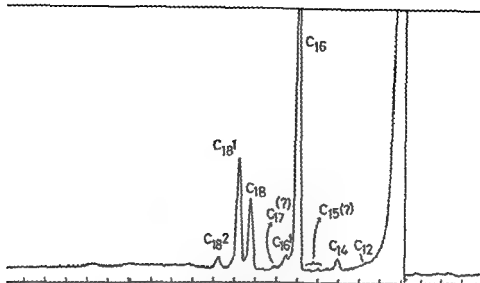


Fig. 3

Gas chromatography of the fatty acids isolated from egg yolk plates after reaction with enzyme

ing at 196–201° C. After treatment with hydrochloric acid, extraction with chloroform and evaporation of the chloroform phase, about 7 mg of the substance was obtained. This contained molten and crystalline solids the melting points of which were at 25, 31–33 and 46–51° C, thus indicating the presence of not less than three major components.

Analysis of fatty acids by gas chromatography. The fatty acids were analysed by gas-liquid chromatography after boron trifluoride methylation. Experiments done with an Aerograph and a Perkin Elmer 800 gas chromatographic instruments gave similar fatty acid distributions. As shown in Fig. 3, the major components in decreasing order were palmitic acid (C_{16}), oleic acid (C_{181}) and stearic acid (C_{18}). The quantitative distribution of these and other fatty acids were as follows:

myristic acid	(C_{14})	1% \pm 0.5%
palmitic acid	(C_{16})	63% \pm 2.0%
palmitoleic acid	(C_{161})	1% \pm 0.5%
stearic acid	(C_{18})	12% \pm 1.0%
oleic acid	(C_{181})	22% \pm 1.5%
linoleic acid	(C_{182})	1% \pm 0.5%
other acids		trace amounts

Thus, the end products of the egg yolk reaction were fatty acids which occurred as insoluble calcium or magnesium salts.

DISCUSSION

The experiments described in this study relate to the identification of the substrate in egg yolk and the products of the egg yolk reaction.

fractionation of egg yolk and examination of the of the fractions for substrate activity showed that the substrate occurred in the lipoprotein fraction. At least two lipoproteins, lipovitellin and lipovitellenin, are known to occur in egg yolk (2). Lipovitellin was shown to contain more phospholipids and less triglycerides, whereas lipovitellenin is not as readily soluble in sodium chloride solution as lipovitellin but dissolves on addition of ether. Further, lipovitellenin has a lower density than lipovitellin. The results obtained in the present study point to lipovitellenin as being the substrate active in the egg yolk reaction. The formation of fatty acids from lipovitellenin by the *Styphlococcus* enzyme indicated that the latter was a lipoprotein lipase. The lack of reaction with lipovitellin was probably due to its lower content of triglycerides. It has been found that the *Styphlococcus* enzyme is not active against phospholipids such as lecithin, cephalin and sphingomyelin (7, 8, 10). It thus seems that the fatty acids were liberated from the triglyceride part of lipovitellenin. Shaw & Wilson (8) also showed that it was lipovitellenin in egg yolk which acted as the substrate in the egg yolk reaction.

The fatty acid distribution indicated that palmitic acid occurred in high amounts followed in decreasing order by oleic and stearic acids. The proportion of the fatty acids reported in the present study did not correspond with the fatty acid distribution described by Martin *et al.* (6). However, the latter writers hydrolysed the total lipids extracted from lipoproteins by chemical means and thus also included the phospholipids. The results obtained in the present study may be due to the relative rates at which the different fatty acid ester bonds were attacked by the enzyme. The greater occurrence of palmitic acid would point to a higher affinity of the enzyme to this ester than to those of oleic or stearic acid esters. A similar observation was also made by Vadehra & Harmon (11) during studies on the action of *Styphlococcus* lipase on milk fat.

SUMMARY

The nature of the egg yolk reaction given by *Styphlococcus* was studied by fractionating egg yolk to identify the substrate and by analysing the products of the reaction. The substrate in egg yolk was identified as lipovitellenin from its low density and ready solubility in the presence of ether. The fatty acids which occurred as insoluble calcium or magnesium salts caused the precipitation reaction. Analysis of the fatty acids gave the following distribution: palmitic acid (63 per cent), stearic acid (12 per cent) and oleic acid 11.5 per cent.

The egg yolk reaction was caused by a lipoprotein lipase acting on lipovitellenin.

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NON-SPECIFIC IMMUNOFLOUORESCENCE STAINING AND ITS ELIMINATION

*Studies on Lymphoid Cells from Rabbits Immunized
with Human Gamma Globulin*

By

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Immunofluorescence methods have been used to demonstrate antigens in tissue or cells and also to demonstrate antibodies present in lymphoid cells (reviewed by Vain 1964). Non-specific staining reactions often complicate immunofluorescence studies. Non-specific interactions between fluorescent material and the cells or tissues studied have attracted most interest. Many investigators have reported success in avoiding the problem by purification of the fluorescent antisera in various ways—for instance, Riggs *et al.* (1960), Urdain (1961), Goldstein *et al.* (1961), Leemhagen & Spendlove (1962), McDevitt *et al.* (1963), Engelgardt (1964), Wolf *et al.* (1965)—or by special treatments of the cell or tissue preparations—for instance, Mayersbach & Schubert (1960). With the indirect fluorescence method staining due to non-specific reactions by the unconjugated middle layer has attracted less interest, although discussed by Myers *et al.* (1965). At a study with immunofluorescence methods on antibody content of lymphoid cells the latter type of non-specific fluorescence became a serious problem and initiated a detailed investigation on such phenomena and on possibilities of eliminating them by various treatments.

MATERIAL AND METHODS

Antigen. A commercial human gamma globulin preparation (AB Labi Stockholm) was used. It is supplied as a 12 per cent solution in a 0.3M glycine bicarbonate buffer at pH 6.8, stated to contain 93 per cent γ_2 and 0.5-1 per cent albumin. An immunoelectrophoretically purer γ_2 preparation (Labi 1348) was used for immunofluorescence and immunodiffusion in agar. This preparation contained except for γ_2 mere traces of albumin.

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Immunization schedule Adult rabbits (2.3 kg) of mixed stock were used. Four groups of rabbits were studied:

1) Untreated called untreated controls—four rabbits
 2) Injected with Freund's complete adjuvant (Difco), called adjuvant treated controls—nine rabbits. One of these was given 0.5 ml intravenously and 0.5 ml intracutaneously in the paws. The other eight were injected intracutaneously with 0.1 ml in equal volumes of saline. The injections were repeated once after 9–10 days.

3) Injected intracutaneously in the paws with 6 mg of human gamma globulin in saline emulsified with an equal volume of Freund's complete adjuvant—15 rabbits. They were given 0.2 ml repeated after 9–10 days. Some animals had repeated injections (up to five) at varying intervals. No material differences in results were seen between the former and the latter rabbits and most studies were performed on the former.

4) Injected intravenously (10 or 20 mg per dose) and intraperitoneally (30 or 60 mg per dose) for a month according to the schedule suggested by Boyd (1956)—nine rabbits.

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 injected intravenously and intraperitoneally no antibodies could be demonstrated with this method some days after the last antigen dose. Sera from all nine intravenously and intraperitoneally injected rabbits were therefore studied with a Coombs test. Human O Rh+ red blood corpuscles were coated with human anti-D serum and tested for agglutination with the rabbit sera before and after the injection schedule. The four rabbit sera that showed precipitates in the immunodiffusions had titres of 1:1024 or 1:2048 and the five rabbit sera without precipitates were negative. These five rabbits are called sero-negative.

Immunodiffusion in agar gel. Rabbit sera before and after injection schedules were analysed with immunodiffusion in agar according to Ouchterlony's micro method with standard equipment from LKB (Stockholm). Undiluted serum was put in the central well and human gamma globulin in the peripheral wells in dilution steps of 1:2 in a wide range of dilutions.

Preparation of lymphoid cells. Cell suspensions were prepared from regional lymph glands and/or the spleen by teasing and the suspension filtered through double gauze and washed three times in saline. A drop of the suspension was placed on a microscope slide. This was put on a sand bath covered with a horizontal glass plate thermostatically controlled at 36°C and the drop of the suspension was dried and fixed in this manner for 20 minutes. A cell density was chosen that permitted a relatively dense cell mat each cell however separated from its neighbours. The slides were stored below -20°C.

From some rabbits lymph glands have been removed more than once. Therefore 11 cell preparations were studied from the nine adjuvant treated rabbits and 16 cell preparations from the 15 rabbits immunized intracutaneously with human gamma globulin.

Reagents used at immunofluorescence. Antiserum globulins conjugated with fluorescein isothiocyanate (FITC) were obtained from Microbiological Associates (Bethesda Maryland): goat antiserum globulin vs human serum globulin (control 43465) goat antiserum globulin vs guinea pig serum globulin (control 44315). Rhodamine conjugated bovine albumin (Microbiol Assoc) was used as counterstain in all conjugated antisera in recommended dosage. Unconjugated goat antiserum vs human serum globulin was obtained from Microbiol Assoc (control 52213).

— — — — — according to Marshall et al
 — — — — — or. However instead of the
 — — — — — used both conjugates con-
 — — — — — gamma globulin was con-
 — — — — — jeine bicarbonate buffer at

pH 6.8
Preincubation and blocking methods. In some experiments human gamma globulin and conjugated antiserum globulin were preincubated with a pool of normal rabbit sera concentrated to a third of its original volume with a collodion bag (Vies 1953). This concentration was found suitable in preliminary experiments.

One part of 12 per cent human gamma globulin and 11 parts of the pool were incubated for 90 minutes at 37°C. The conjugated commercial antiserum of human gamma globulin was mixed with an equal volume of the pool and treated in the same way. Precipitates were not formed in any of these incubations.

In other experiments preincubation was performed with equivalent amounts of rabbit gamma globulin prepared by free electrophoresis in a 0.05M Tris HCl buffer at pH 8.0. Layer electrophoresis showed it to be made up of 99.3 per cent gamma globulin and 1.7 per cent beta globulin.

In further experiments conjugated anti-human globulin was blocked with the antigen by incubation for two hours at 37°C. Two parts of the conjugate and one part of 12 per cent human gamma globulin were used. After incubation the mixture was centrifuged (one part of 12 per cent human gamma globulin was again added and incubation was continued for 12 hours at +4°C. After centrifugation preincubation as above with concentrate of pooled rabbit sera was performed. Parallel controls with 0.9 per cent saline instead of human gamma globulin were made.

Immunofluorescence method. Various modifications were used as further described under Results. The first step (incubation with antigen or control) was made with a volume of 1 or 3 drops at 37°C for 30 minutes. When intermediate step was used the slides were incubated with 5 drops of unconjugated goat serum at 37°C for 45 minutes. This procedure was repeated once or twice. The final step (conjugated antiserum of human gamma globulin) was made at room temperature for 15 minutes with a volume corresponding to two drops of unlabeled conjugate. As rinsing solution Sørensen's 0.15M phosphate buffer at pH 7.2 or saline buffered at pH 7.1 with 0.01M phosphate were used. The two solutions were found equivalent.

Microroscopy was performed with a Zeiss II fluorescence microscope equipped with an Osram high pressure Mercury arc lamp HBO 200 Bc 3 and Hg 12 exciter filters and a barrier filter 44 and 50 ("cut off" at 360 mμ). A 40× objective was used. The microscope could easily be switched to phase contrast. Photographs could be taken on 35 mm High Speed Ektachrome film Ektab 135 20.

The slides were read blind by two different observers. Usually a positive control was included in the series of slides studied.

The fluorescence intensity was scored from (+) to ++. Positive slides are of two types: in one practically every cell is fluorescent with scores from (+) to ++ or ++ (cf. Fig. 2). In the other type single fluorescent cells with a reaction of at least ++ are observed in a negative (cell shadow) background (cf. Fig. 1). For further discussion in the scoring of positive slides see § 3rd.

RESULTS

1. Direct Fluorescence Method

The simplest way to demonstrate the presence of cells with antibodies against human gamma globulin would be to use the direct immunofluorescence method. One per cent conjugated human gamma globulin in saline was added to slides from five rabbits. Two untreated controls, one adjuvant treated control and two "sero-positive" rabbits. In all these slides a generalized fluorescence of equal strength was obtained (see Fig. 2).

Thus conjugated human gamma globulin becomes attached to cells of all types and reacts with the antigen. The reaction is not dependent on the type of cell, but on the presence of the human gamma globulin. The reaction is not dependent on the type of cell, but on the presence of the human gamma globulin.

Human gamma globulin was preincubated with concentrated rabbit serum (cf. "Methods") and added to the five cell types.



Figs 1 and 2

Fig 1 Colour photograph of lymphoid cells from a rabbit immunized intracutaneously with human gamma globulin. Indirect fluorescence method: first step, human gamma globulin preincubated with normal rabbit serum; final step, fluorescein-conjugated goat anti human globulin preincubated with normal rabbit serum. Four fluorescent cells scored (+) to ++ in a negative background.

Fig 2 Colour photograph of lymphoid cells from a rabbit injected intracutaneously with Freund's complete adjuvant. Direct fluorescence method: treatment of slides with fluorescein-conjugated human gamma globulin. Note generalized fluorescence.

parations mentioned above. No fluorescence could now be seen either in cells from controls or in cells from "sero-positive" rabbits. Thus either no specific attachment of human gamma globulin to cells from immunized animals occurred or the direct method used is too insensitive for its demonstration. An indirect method was therefore tried.

2. Indirect Fluorescence Method

Slides from three untreated and five adjuvant treated controls were exposed to one per cent human gamma globulin in saline in the first step and conjugated goat anti human globulin diluted in an equal volume of saline in the second. A generalized fluorescence was obtained in all slides similar to that shown in Fig. 2. This could not be due to a non specific attachment of FITC or FITC conjugated material to the cells as slides from these cell preparations were negative when exposed to saline in the first step without changing the second step. The result indicates attachment of the unconjugated human gamma globulin to the cells in the first step.

However FITC or FITC-conjugated material added in the second step may sometimes react with the cells. This interaction varies in strength for different conjugates as well as for different cell preparations which the following experiments show. Slides from two untreated and five adjuvant treated controls also studied with conjugated goat anti human globulin as described above were treated with human gamma globulin or saline in the first step and conjugated goat anti human globulin diluted in an equal volume of saline in the second. Five cell preparations were negative but two showed single positive cells in a negative background both when the first step consisted of human gamma globulin and when it consisted of saline. Among slides from three "sero-negative" rabbits studied with saline in the first step and conjugated goat anti human globulin diluted in an equal volume of saline in the second one showed a similar type of fluorescence resembling that shown in Fig. 1.

As it was demonstrated with the direct method that pretreatment of conjugated human gamma globulin with normal rabbit serum concentrate diminished or extinguished its absorption to the cells, the same schedule was tried in the indirect method. In the first step slides were treated with human gamma globulin that had been preincubated with concentrated normal rabbit serum. In the final step conjugated goat anti human globulin in an equal volume of saline was used. Slides from one untreated and two adjuvant treated rabbits all showed a generalized fluorescence.

Although this generalized fluorescence was a little less pronounced than before this simple pretreatment obviously will not prevent non specific fluorescence. Pretreatment with concentrated normal rabbit serum was therefore tried both in the first and the second step. 20 11

preparations from untreated controls, adjuvant-treated controls or "sero-negative" rabbits were studied in this way—all were now negative. The same result was obtained with these cell preparations when the first step consisted of concentrated normal rabbit serum and the second step of conjugated goat anti-human globulin, pretreated with normal rabbit serum concentrate. Thus, control cell preparations were now negative.

Subsequently, 20 cell preparations from "sero-positive" rabbits were studied in the same way. After pretreatment with normal rabbit serum concentrate in both steps, scattered strongly fluorescent cells in a negative background (see Fig. 1) were seen in 18 preparations. One of the two negative preparations were obtained from a regional lymph node, removed less than one day after the booster dose, given on day 10. The other was obtained from a spleen removed five days after the last injection to a rabbit, immunized intravenously.

19 cell preparations from "sero-positive" rabbits were studied with concentrated normal rabbit serum in the first step and conjugated goat anti-human globulin, pretreated with concentrated normal rabbit serum, in the second step. 18 of these were negative, but in one, scattered fluorescent cells were observed on a negative background. This preparation was obtained from regional lymph gland and spleen, removed four days after the booster dose, given on day 10. Further analysis of this cell preparation (see below, p. 327) showed that this reaction is reasonably immunologic. This can be explained by a supposed presence of antigen in the cells. It is surprising that such findings were not obtained more frequently.

Thus, in the main, cell preparations from controls are now negative and from "sero-positive" rabbits positive. No generalized fluorescence is observed. Positive slides show many strongly fluorescent cells on a negative background. It should be stressed, that a positive reaction is scored when the fluorescent cells are fairly numerous and show a strength of at least + (+). Such single cell in a whole slide is judged to be without significance, like cells with a fluorescence of (+) up to + strength.

■ *Specificity of Reactions*

The specificity of the positive reactions in cell preparations from "sero-positive" rabbits were now further studied using three different systems.

In the first series of slides, a conjugated goat anti guinea-pig globulin was used in the final step instead of the anti-human globulin. Preincubation with normal rabbit serum concentrate was performed with both the human gamma globulin of the first step and with the conjugate of the final step. Negative results were obtained with 20 cell preparations from "sero positive" rabbits.

In the second series of slides the conjugated goat anti human globulin was pretreated with an excess of human gamma globulin in order to block specific antibodies. This blocked conjugate was then used in the final step after preincubation with concentrated normal rabbit serum the first step being treatment with human gamma globulin also preincubated with rabbit serum concentrate. Three "sero-positive" rabbits showed negative slides. When conjugate was "blocked" with saline instead of human gamma globulin however, positive slides were obtained in identical preparations.

In the third series the slides were first treated in the usual way with human gamma globulin preincubated with rabbit serum concentrate. An intermediate step was then made with unconjugated goat anti human globulin followed by a final step of treatment with conjugated goat anti human globulin preincubated with normal rabbit serum concentrate. Three "sero-positive" rabbits the same as used in the second series mentioned above showed negative slides in this system.

The third system however might have been complicated by a non specific blocking effect of the goat serum in the intermediate step. Therefore a control series was run with the same cell preparations using normal goat serum in the intermediate step the rest of the experiment being unchanged. Among the three cell preparations studied only one showed an uncertain positive reaction the other two being negative. Therefore the blocking effect of the unconjugated anti serum may not necessarily demonstrate an immunologic specificity but may be a non specific effect between goat serum and human gamma globulin. In the present experimental system the use of unconjugated antiserum in an intermediate step usually regarded as a reliable control of specificity is thus dubious.

When non specific attachment of human gamma globulin in the first step to cells from control rabbits is investigated with the indirect method a test of specificity of its second step is necessary. This was already partly done using conjugated goat anti human gamma globulin (see p. 324). Further controls were obtained by blocking the conjugated goat anti human globulin with human gamma globulin. The blocked conjugate was now pretreated with concentrated normal rabbit serum and used for the final step the first step being treatment with human gamma globulin in saline. Cell preparations from two untreated controls and three "sero-negative" rabbits showed no fluorescence after this treatment—when blocking was made with saline all showed a strong generalized fluorescence similar to that shown in Fig. 2.

Finally the specificity of the reaction found in one rabbit was analysed. Its cells gave a positive fluorescence after a first step incubation with concentrated normal rabbit serum and a final step with conjugated goat anti human globulin pretreated with concentrated normal rabbit serum (see p. 326). Blocking of the conjugated antiserum globulin with an excess of human gamma globulin gave negative reaction

Also when conjugated goat anti-guinea-pig globulin was used in the final step, a negative result was obtained. Thus, also this reaction is apparently specific.

4 Further Technical Studies

In order to study different test systems, as outlined above, it was necessary to keep slides with cell films frozen for a considerable time. The effect of the freezing and of storing below -20°C on the outcome of specific and non-specific fluorescence reaction was explicitly studied.

Two series of slides from rabbits injected with Freund's complete adjuvant were studied for non-specific generalized fluorescence with the indirect method: a first step with human gamma globulin in saline and a final step with conjugated goat anti human globulin in saline. Slides were studied before freezing, after one day of storage in deep-freeze, and after one and two months of storage. Representative colour pictures were taken on each occasion. The non specific fluorescence did not change.

Slides from 11 rabbits immunized with human gamma globulin and showing specific positive cells were also studied with the indirect method: human gamma globulin preincubated with concentrated normal rabbit serum as a first step, and conjugated goat anti-human globulin preincubated with rabbit serum concentrate as the final step. Any differences were not found before and after freezing, nor after storage for up to one year.

Thus, freezing and storage do not influence the result of the fluorescence technique.

For preincubation of the human gamma globulin with normal rabbit serum a one per cent solution of human gamma globulin in a concentrated pool of normal rabbit sera (cf "Methods") was used. In some cases, to save concentrated rabbit serum a 0.25 per cent solution of human gamma globulin in unconcentrated pooled normal rabbit serum was used and the conjugated goat antiserum globulin was now also preincubated with an equal volume of unconcentrated normal rabbit serum. Comparison between these two modifications usually showed no difference.

DISCUSSION

In the present paper, possibilities of demonstrating with immunofluorescence methods antibody-containing lymphoid cells taken from rabbits immunized with human gamma globulin is analysed. The direct immunofluorescence method used is not sufficiently sensitive for this purpose but the indirect method can be used. The observations are complicated by a number of non-specific reactions. The observable conjugated goat anti-human globulin may be attached to the cells in various ways.

a) A specific immunologic reaction obtained by a coupling of the fluorescent antibody to human gamma globulin specifically combined with those lymphoid cells that contain antibodies against human gamma globulin. This is the desired reaction.

b) Fluorescent components, possibly although not necessarily antibody, may become attached to the cells without any interaction with human gamma globulin. This phenomenon is a well known complication in immunofluorescence studies and has been attacked in different ways for instance by various treatments of the conjugate (cf. Introduction). It was observed with different strengths in cell preparations from different rabbits. The presence of normal rabbit serum in the first step strengthens this type of fluorescence (cf. *Heutner 1961* also *Mayerstach & Schubert 1960*).

c) The lymphoid cells may contain human gamma globulin given as antigen and therefore directly react with the conjugated goat anti human globulin. This was seen only once (see p. 326).

d) A non specific coupling of human gamma globulin to rabbit cells is a consistent and pronounced phenomenon. At indirect immunofluorescence this phenomenon will cause a generalized fluorescence due to the non specific reaction between the unconjugated middle liver and the cells. As mentioned in the introduction little interest has been paid to this subject in the literature. This attachment of human gamma globulin to the rabbit cells could be the result of a non specific protein absorption, or it could represent an immunologic reaction between the human gamma globulin and antigens present in the rabbit cells. These antigens may cross react with some unknown antigen that has resulted in an antibody formation in man. The present author finds the former explanation more likely.

e) Theoretically the goat anti human or anti guinea pig globulin can react immunologically with rabbit protein on the cell surface and produce fluorescent staining. Either the conjugated goat antiserum globulins used may be contaminated by traces of anti rabbit serum or rabbit proteins (e.g. gamma globulin) may have antigens common with human and guinea pig serum globulin.

Schematic illustrations of these five alternatives are given in Fig. 3.

Two of these reactions (b, d) can be blocked by pretreating the human gamma globulin and the conjugated goat antiserum globulin with normal rabbit serum. A component common to serum and lymphoid cells is thus reasonably responsible for this non specific binding. Therefore a trial was made to block the reactions by preincubation with rabbit gamma globulin. A solution of 36 mg/ml in saline was used because it was supposed to correspond to the gamma globulin concentration found in a concentrated serum (normal gamma globulin concentration in rabbits is given by *Andersen & Bjørnøe 1964*). This was studied on cells from one normal rabbit and four rabbits immunized with human gamma globulin in previous study.

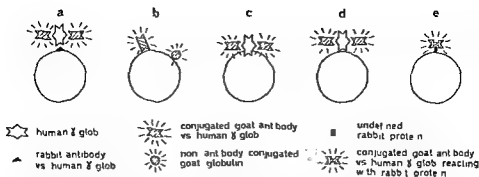


Fig 3

Schematic drawings illustrating five possible methods of staining with fluorescein conjugated goat antiserum globulin with lymphoid cells at indirect method a specific fluorescence, caused by the presence in the cells of antibodies against human gamma globulin b non specific fluorescence, caused by direct coupling between cell and fluorescent component in goat antiserum globulin c fluorescence caused by the presence of human gamma globulin as an antigen in the cell d fluorescence, caused by non specific absorption of human gamma globulin to the cell, the final step being specific + fluorescence caused by immunologic reaction between conjugated goat antiserum globulin and rabbit protein Further explanation in text

found to show a specific positive reaction No difference could be found between slides made with rabbit gamma globulin and those made with normal rabbit serum as preincubation medium Thus, the gamma globulin of normal rabbit serum has the same capacity of blocking non specific reactions as whole serum, it cannot be excluded that other serum components have the same effect The hypothesis that the non-specific reactions were due to non immunologic coupling of gamma globulins from the different species used seems reasonable

SUMMARY

Non specific staining reactions at immunofluorescence were studied using lymphoid cells from rabbits immunized with human gamma globulin and from various control rabbits

When the direct method was used, conjugated human gamma globulin formed a non-specific attachment with lymphoid cells, irrespective of the source of origin This did not occur when the human gamma globulin was pretreated with normal rabbit serum This method, however was too unsensitive to permit visualization of antibody-containing cells from immunized rabbits

With the indirect method, including treatment of the cells with unconjugated human gamma globulin followed by fluorescein conjugated goat anti-human globulin, a generalized non specific fluorescence occurred This was mainly due to a non specific attachment of the unconjugated human gamma globulin to the cells This interaction in the middle layer was eliminated when the human gamma globulin was pretreated with normal rabbit serum Also the conjugate

of the second step could react non specifically with the cells. This reaction did not occur when also the conjugate was treated with normal rabbit serum. The pretreatment of both steps eliminating both types of non specific fluorescence did not prevent the specific fluorescence obtained by reaction of human gamma globulin with antibody containing lymphoid cells from immunized rabbits.

Rabbit gamma globulin can be substituted for rabbit serum at pretreatments. This suggests by way of explanation of the non specific staining an interaction between cellular rabbit gamma globulin and human perhaps also goat gamma globulin added to the cells.

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INDUCTION OF THYROIDITIS IN GUINEA PIGS BY SERUM FROM RABBITS IMMUNIZED WITH GUINEA PIG THYROGLOBULIN

Eu

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Ever since the demonstration of precipitating antibodies against human thyroglobulin in serum of patients suffering from Hashimoto's disease (21) and the production of thyroglobulin antibodies and thyroiditis by immunization with homologous thyroid extracts in experimental animals (28, 24), the pathogenic capacity of thyroid antibodies in serum has been questioned.

The problem has been approached in several ways

- 1 Transfer of serum from individuals with thyroiditis to normal recipients Rott & Doniach (20) transferred serum from patients with high levels of thyroid antibodies to monkeys However no functional or morphological alterations of the monkey's thyroid could be detected Rose Aite & Hoebbler (24) failed to transfer thyroiditis by repeated injections of rabbit anti rabbit thyroid immune serum into healthy rabbits
 - 2 Studies on the offspring of females with thyroid antibodies in serum Although thyroid antibodies cross the placenta barrier no functional or histological damage has been observed in the thyroid of newborn children (19) rabbits (2) or guinea pigs (25)
 - 3 Segregation of thyroiditis and thyroid antibodies Thyroiditis may be produced in guinea pigs without induction of serum antibodies (17) On the other hand thyroid antibodies may be induced without histological alterations of the gland (23)
 - 4 Lack of course a delayed antibody • - • thyroiditis The closely related to a serum thyroid
- Transfer of thyroid immune serum produced by heterologous immunization Rott Jones & Doniach (22) injected serum from rabbits immunized with rat thyroglobulin into rats No damage of the recipient's thyroid was observed when serum was given alone Animals previously exposed to ionizing radiation or Freund adjuvants however revealed thyroiditis within a few days after intraperitoneal injections of thyroid immune serum The histological alterations apparently consisted of infiltration of the thyroid by mononuclear cells

The present investigation followed the last approach. As recipients guinea pigs were employed with the rabbit as the antibody producing species.

MATERIALS AND METHODS

Animals. Randomly bred white female guinea pigs strain AC with body weights of 250-350 grams were obtained from the National Institute of Public Health. Old and very old rabbits initially weighing 2000-3000 grams were employed for production of antiserum.

Antigens. Thyroid glands of CO₂-killed and desanguinated guinea pigs were excised, trimmed of fat and cut into small pieces. After addition of 2 volumes saline (w/v) the mixture was homogenized at 0°C and stirred at 4°C overnight. After centrifugation (4000 × g × 30 minutes) the supernatant was used for separation of thyroglobulin. Thyroglobulin was prepared by (NH₄)₂SO₄ fractionation between 30 and 50 per cent saturation with subsequent dialysis against running water for 24 hours, distilled water for 24 hours and saline for 24 hours. The thyroglobulin preparation was stored at -20°C.

Extracts of guinea pig kidney, liver and spleen were prepared in an identical manner. After centrifugation (4000 × g × 30 minutes) the supernatant was employed for immunization and gel precipitation studies.

Antisera. Rabbits were immunized with guinea pig thyroglobulin or guinea pig kidney extracts. Five mg of antigen per ml of saline was mixed carefully with equal volumes of Difco's Freund's complete adjuvants. 0.6 ml of the mixture was injected as superficially as possible in all 4 foot pads once a week for 4 weeks. The rabbits were bled 3 times during the second week after the last injection and the serum obtained was pooled. Two months later the animals were bled serially, one injection into each foot pad and bled a second time. Guinea pigs were immunized in a similar way with rabbit serum by injection of 0.025 ml into each of the foot pads.

Immiprecipitation in gel. The method of Ouchterlony (18) as described by Friksen (3) was employed. Constituents of the gel were 0.03 M barbital buffer at pH 8.6, 0.003 per cent methyl orange, 1 per cent Difco Special Agar No. 1 and 0.1 per cent phenol.

Hemagglutination. The indirect hemagglutination procedure of H. A. M. Allen (4) was employed. Human erythrocytes were formalinized and sensitized with guinea pig thyroglobulin as previously described (5, 6, 7) with employment of formal guinea pig serum instead of formal rabbit serum as stabilizer. The concentration of antigen used for sensitization was 0.5 mg per ml against 1.2×10^8 cells per ml. Tanned, uncoated erythrocytes were used for control of specificity. The sera were screened in dilutions 1:5, 1:25, 1:250, 1:500 etc. with twofold titration of intervals when a more precise titer was desired.

a. *serum* (by the direct method)

the killed and desanguinated

These sections were either stained with hematoxylin and eosin or by the May-Grunwald and Giemsa techniques.

RESULTS

Specificity of Rabbit Antisera

Serum of rabbits immunized with guinea pig thyroglobulin or kidney extract appeared to react with guinea pig serum in Ouchterlony plates. Consequently, rabbit immune serum was absorbed with guinea pig serum. Absorption was performed at 4°C in several steps by using guinea pig serum: rabbit antiserum ratios of 1:10, 1:10 and 1:2. The anti-guinea pig thyroglobulin serum did then not react with guinea pig plasma as shown in Fig. 1. Rabbit immune serum against guinea pig kidney had to be absorbed 1:10, 1:10, 1:2, 1:2 and 1:2 before all antibodies against guinea pig plasma were removed as tested by precipitation in gel (Fig. 1). Thyroglobulin antiserum was found to react with a single line against preparations of guinea pig thyroglobulin as well as whole thyroid extracts but did not react with extracts



Fig 1

thyroid extract (5 mg protein per ml) 6 Guinea pig thyroglobulin (5 mg per ml)

guinea pig liver, spleen or kidney. On the other hand, immune serum against guinea pig kidney gave rise to 2 lines against kidney extracts but did not react with thyroglobulin or thyroid extracts.

The absorbed thyroglobulin immune serum gave rise to a hemagglutination titer of 1:100,000 by cells coated with guinea pig thyroglobulin.

Histologic Alterations in the Thyroid Gland of Guinea Pigs Following Intraperitoneal Application of Rabbit Immune Serum Against Guinea Pig Thyroglobulin

As shown in Fig 3a as compared to Fig 2, remarkable histological changes were found in the thyroid gland of guinea pigs injected 24 hours previously with thyroglobulin immune serum. In histological sections confluent infiltrations of polymorphonuclear leukocytes were observed. The granulocytes were mainly located interstitially but rupture of follicles with streaming of granulocytes into the colloid was frequently seen (Fig 3b). Proliferation of histiocytes seemed not to be predominant at that time and occasionally only, lymphocytes were observed. Blood vessels appeared normal. The granulocytes revealed rod shaped or segmented nuclei with 2-3 globules, and their cytoplasmic granules were stained slightly eosinophilic by hematoxylin and eosin (Fig 3b) and red by the May-Grünwald and Giemsa staining (Fig 3c). Blood smears stained by the May Grünwald and Giemsa technique were found to contain less than 1 per cent of such cells while approximately 50 per cent of the leukocytes consisted of granulocytes with hypersegmented nuclei and with small and poorly stained cytoplasmic granules (Fig 4). These observations would seem

TABLE 2
Histological and Serological Findings at the Time of Sacrifice in Guinea Pigs Receiving Intraperitoneal Injections of Bivalent Anti-Guinea Pig Immune Serum

Animal no.	Serum injected	Volume (ml) injected	Time of sacrifice	Hemagglutination titer of thyroglobulin at the time of sacrifice	After adsorption with guinea pig anti-rabbit serum	Severity of histological lesion in the thyroid
1	anti-thyroglobulin	4	24 hours	1:20,000	—	++
2			"	1:10,000	—	++
3			48 hours	1:5,000	—	0
4	"	"	"	1:20,000	—	+
5			5 days	1:10,000	—	++
6			"	1:10,000	—	++
7			10 days	1:5,000	<1:25	+
8	"		"	<1:5	—	0
9	"		20 days	1:25	<1:5	0
10	"		"	1:1,000	<1:25	+
11	anti-guinea pig kidney extract		24 hours	<1:5	—	0
12	"		"	<1:5	—	0
13	"		5 days	<1:5	—	0
14	"		"	<1:5	—	0
15			10 days	<1:5	—	0
16			"	<1:5	—	0
17			20 days	<1:5	—	0
18			"	<1:5	—	0

— none

Grade according to Jones & Koelt (10) + — separate foci of inflammatory cells + + — confluent inflammatory cell infiltration of thyroid architecture
+ + — confluent infiltration of inflammatory cells with destruction of follicular architecture

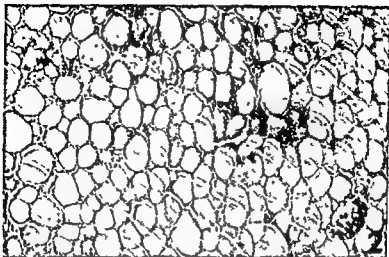


Fig 2

Normal appearance of a guinea pig thyroid (animal 3-11),
(hematoxylin and eosin $\times 70$)

to indicate that the granulocytes found in the thyroid of guinea pigs receiving thyroglobulin immune serum are eosinophils

Five days after the injection of immune serum, there were still confluent infiltration of granulocytes, but the dominant cell type at that time was the histiocyte (Fig 5 a and b). There was a moderate increase in the number of lymphocytes.

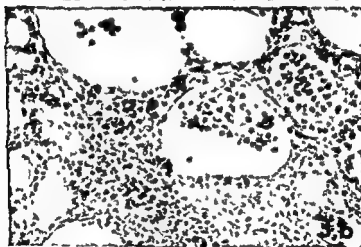
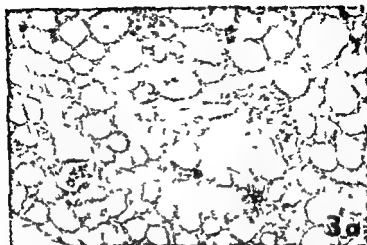
Ten or 20 days after the injection of immune serum, the histologic alterations of the thyroid had regressed and changed in character. Only 2 out of 4 animals showed a mild degree of thyroiditis (Table 1). The thyroid of the other 2 animals appeared normal. Although granulocytes still could be observed, they were mainly located intrafollicularly. Small groups of lymphocytes and slight fibrosis were now seen in the interfollicular spaces (Fig 6).

None of the animals injected with rabbit immune serum against guinea pig kidney revealed histological changes of their thyroid (Fig 2) and no pathological changes could be observed in the liver or kidney of any animal.

Most of the animals receiving thyroglobulin immune serum on the day of sacrifice appeared to have large quantities of circulating rabbit

Fig 3

Thyroiditis (grade ++) in a guinea pig 24 hours after injection of rabbit anti guinea pig thyroglobulin (animal 3-11). a) Interstitial and follicular invasion of (hematoxylin and eosin $\times 70$) b) predominantly polymorphonuclear leukocytes (hematoxylin and eosin $\times 175$) c) which are stained red by May Grunwald and Giemsa (May Grunwald and Giemsa $\times 175$)



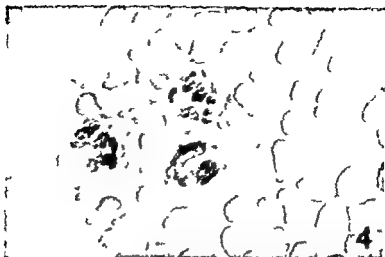


Fig 4

A blood smear stained by May Grunwald and Giemsa showing 1 cell with a nucleus consisting of 2 globules and with a cytoplasm rich in red coloured granules. Less than 1 per cent of the leukocytes consisted of such cells. The other 2 cells found in a frequency of about 50 per cent have a hypersegmented nucleus with few and poorly stained cytoplasmic granules (May Grunwald and Giemsa $\times 1080$)

antibodies against guinea pig thyroglobulin (Table 1). Even in animals sacrificed 10 or 20 days after injection of rabbit immune serum all the thyroglobulin antibodies were removed by absorption with guinea pig immune serum against rabbit serum (Table 1). Apparently all thyroglobulin antibodies left in serum were of rabbit origin.

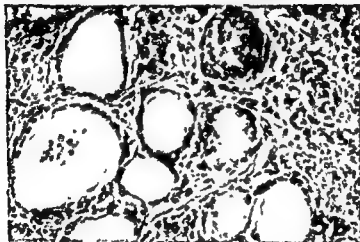
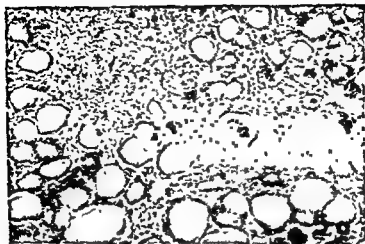
DISCUSSION

The present study has shown that heterologous immune serum against thyroglobulin may affect the thyroid of healthy guinea pigs. These results are at variance with those of *Ittli, Jones & Doniach* (22) who observed effects of heterologous immune serum only on the thyroid of rats pretreated with ionizing radiation or Freund adjuvants. Pretreated animals revealed a type of thyroiditis similar to the inflammation which appears after active immunization with mixtures of thyroid extracts.

Figs 5-6

Fig 5 Thyroiditis (grade +++) in a guinea pig 5 days after receiving rabbit anti guinea pig thyroglobulin (animal 5-6). a) Confluent infiltration with destruction of follicular architecture (hematoxylin and eosin $\times 70$). b) Poly mononuclear cells predominantly of the histiocyte type, some follicles still contain granulocytes (hematoxylin and eosin $\times 175$).

Fig 6 Thyroid of a guinea pig 20 days after receiving rabbit anti guinea pig thyroglobulin showing small interstitial infiltrations of lymphocytes and granulocytes intracellularly (animal 5-10) (hematoxylin and eosin $\times 70$).



or thyroglobulin in Freund adjuvants. The fast type of thyroiditis may in early stages occasionally reveal infiltration of granulocytes (10, 13, 26, 29), but the dominant inflammatory cell types are lymphocytes and histiocytes.

Although the present type of thyroiditis contains the same elements, the quantitative differences are remarkable. Dominant in the present inflammation is the rapid infiltration of granulocytes which seems to be eosinophils, while the delayed aggregation of lymphocytes is little pronounced.

The mechanism behind this reaction at present remains unknown. Since the reaction is limited to the thyroid and specific to rabbit immune serum against guinea pig thyroglobulin, the factor responsible most likely is a thyroglobulin antibody. Serological studies support this hypothesis (Fig. 1).

Since Litt (14) has shown that eosinophils may be attracted by antigen-antibody complexes, the explanation may be that thyroglobulin antibody complexes attract the granulocytes which seem to be eosinophils. As is the case with classical experimental thyroiditis, most of the inflammatory cells are located interstitially, although the colloid of several follicles may be invaded by inflammatory cells. This aggregation in the interfollicular spaces may be due to the presence of small quantities of thyroglobulin in the interstitial spaces or, the follicle wall may represent a barrier which is difficult for the leukocytes to penetrate. Whether the streaming of cells into the colloid has relation to colloidophagy (8), at present remains unknown.

The delayed infiltration of lymphocytes in the present type of thyroiditis could be a part of an immunological reaction against foreign antibodies in parallel with the Masugi type of nephritis (12). However, since animals with the highest titers of rabbit thyroglobulin antibodies revealed infiltration of mononuclear cells, while animals in which these antibodies had been remarkably reduced did not show infiltration of such cells in their thyroid, the delayed aggregation of lymphocytes may not be a part of an immunological response against the foreign antibodies.

Besides the Masugi nephritis (15), which involves the delicate vascular structures of the glomerulus, previous studies have indicated that serum antibodies only may affect parenchymatous tissues when animals are given certain kinds of pretreatment (22) or when it is applied locally (9). The present study shows that heterologous serum antibodies in healthy recipients through the circulation may reach extravasally located antigens of parenchymatous tissues and initiate an inflammatory reaction.

SUMMARY

Histological changes in the thyroid of guinea pigs following intraperitoneal injection of rabbit anti guinea pig thyroglobulin immune serum

are described. Within 24 hours thyroiditis develops with a massive infiltration of polymorphonuclear leukocytes which seem to be eosinophils. The acute stage is succeeded by proliferation of mononuclear cells, mainly histiocytes, and by a less pronounced delayed aggregation of lymphocytes.

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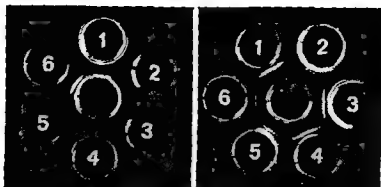


Fig 1

Fig 2

- Fig 1** Precipitation in gel by guinea pig immune sera and guinea pig tissue antigens. Central well: Guinea pig anti guinea pig thyroglobulin (antiserum B A₁ or A H). 1-4: Guinea pig anti Freund adjuvants (antiserum C). 5: Guinea pig liver extract (5 mg protein per ml). 6: Guinea pig kidney extract (5 mg protein per ml). 3: Guinea pig thyroid extract (5 mg protein per ml). 6: Guinea pig thyroglobulin (5 mg per ml).
- Fig 2** Precipitation in gel by heterologous and homologous antisera against guinea pig thyroglobulin and guinea pig thyroglobulin. Central well: Guinea pig thyroglobulin (5 mg per ml). 1-4: Guinea pig anti guinea pig thyroglobulin type B (or A₁). 2-5: Guinea pig anti guinea pig thyroglobulin type A-H. 3-6: Rabbit anti guinea pig thyroglobulin absorbed with guinea pig serum (diluted 1:5).

RESULTS

Specificity of Antisera

As demonstrated in Fig 1 thyroglobulin antisera (A₁, B or A-H) gave rise to an identity reaction between thyroglobulin and an unfractionated thyroid extract with a single precipitin band. No reaction was observed against extracts of guinea pig kidney or liver. Furthermore the various types of antisera between themselves also revealed an identity reaction against thyroglobulin (Fig 2). This identity reaction was extended to include rabbit anti guinea pig thyroglobulin immune serum (Fig 2) which previously has been employed for induction of thyroiditis (2). Serum C failed to react with any of the guinea pig tissue extracts (Fig 1).

Histological Changes in the Thyroid of Guinea Pigs Following Intraperitoneal Injection of Guinea Pig Anti Guinea Pig Thyroglobulin Immune Serum

The histological alterations present at various intervals after a single intraperitoneal injection of 16-20 ml of homologous thyroglobulin immune serum are summarized in Table 1. Twenty-four hours after injections of antiserum A₁ or B, confluent infiltrations of granulocytes throughout the thyroid were observed (Fig 3a). By the May-Grunwald and Giemsa technique the cytoplasmic granules of the polymorphonuclear leukocytes were stained red and their nuclei were rod-shaped.

TABLE 1

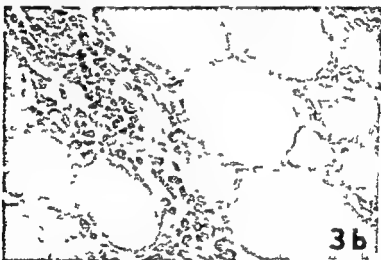
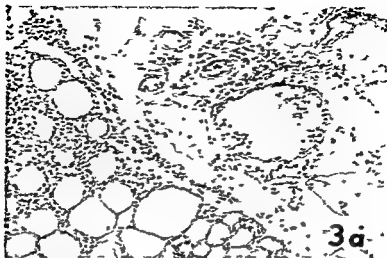
Histological Findings at the Time of Sacrifice in Guinea Pigs Receiving Intraperitoneal Injections of Guinea Pig Anti Guinea Pig Thyroglobulin or Guinea Pig Anti Freund's Adjuvant Immune Serum

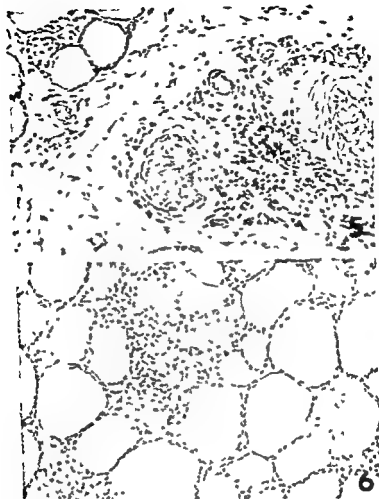
Animal no	Type*	Antiserum injected		Time of sacrifice	Hemagglutination titer of thyroglobulin antibodies	Hemagglutination titer of thyroglobulin antibody at sacrifice	Histological findings in the thyroid	
		Volume (ml)					Severity of lesion	Infiltration of granulocytes
Z-7	A ₁	16		24 hours	1:1000	1:250	++	Interstitial an [†]
Z-8	"	16		24 hours	1:1000	1:200	++	capular [†]
AB-5	B ₁	20		24 hours	1:500	1:100	++	"
Z-9	A ₁	16		5 days	1:1000	—†	+	"
Z-10	"	20		5 days	1:1000	1:250	+	"
AB-6	B ₁	20		10 days	1:500	—	0	0
AB-7	B ₁	20		20 days	1:500	1:5	0	0
AB-4	A ₁	25		20 days	1:1000	1:5	0	0
AB-1	"	25		30 days	1:1000	<1:5	0	0
AB	"	25		50 days	1:1000	<1:5	0	0
AB-8	A ₁ -H	20		24 hours	1:2500	1:200	+	capular [†]
AB-9	"	20		5 days	1:2500	1:500	+	"
AB-10	"	20		10 days	1:2500	1:250	+	"
AB-14	A ₁ -I	20		24 hours	<1:25	<1:5	+	0
AB-15	"	20		5 days	1:25	1:5	+	0
AB-11	"	20		10 days	<1:25	<1:5	0	0
AB-11	C	20		24 hours	<1:5	<1:5	+	0
AB-12	III	20		5 days	<1:5	<1:5	0	0
AB-13	C	20		10 days	<1:5	<1:5	0	0

* A₁ or B Guinea pig anti guinea pig thyroglobulin immune serum obtained after 1 month of immunization with Thyroglobulin immune serum from guinea pigs of group A responding to booster injections 4 months after the first injection of immunized animals. B Serum from animals of group A which failed to respond to thyroglobulin by booster injections. C Anti Freund's adjuvant immune serum.

† graded according to Jones & Roitt (6) + — separate foci of inflammatory cells, ++ — confluent infiltration of inflammatory cells

— not done





Figs 5 & 6

- Fig 5** Thyroiditis (grade +) in a guinea pig 5 days after injection of guinea pig anti guinea pig thyroglobulin (antisera 4; animal 7 10) showing aggregation of granulocytes around vessels adjacent to the thyroid ("capsular" infiltration) (May Grünwald and Giemsa $\times 70$)
- Fig 6** Thyroiditis (grade +) in a guinea pig 21 hours after injection of guinea pig anti Freund adjuvants (animal AB 11) showing an inflammatory focus consisting of lymphocytes and histiocytes (hematoxylin and eosin $\times 70$)

Figs 7 & 8

- Fig 7** Thyroiditis (grade ++ in a guinea pig 24 hours after injection of guinea pig anti guinea pig thyroglobulin (antisera 1; animal AB 5) a) Interstitial and "capsular" infiltration of rat eosin and eosin $\times 70$ b) by predominantly polymorphonuclear cells with a hyposegmented nucleus and a cytoplasm rich in granules (May Grünwald and Giemsa staining (May Grünwald and Giemsa $\times 175$)
- Fig 8** Thyroiditis (grade +) in a guinea pig 5 days after injection of guinea pig anti guinea pig thyroglobulin (antisera 4; animal 7 9) showing interstitial infiltration of granulocytes (May Grünwald and Giemsa $\times 17$)

or consisted of a few globules (Fig 3 b) On this basis, these cells are suspect of being eosinophils (2) Exceptionally only, inflammatory cells were observed intrafollicularly

Five days after injection of thyroglobulin immune serum A₁ or B, the number of granulocytes had decreased without any marked increase of histiocytic elements or lymphocytes (Fig 4) The aggregation of granulocytes adjacent to the thyroid was at that time remarkable ("capsular infiltration") No animals sacrificed 10-50 days after injection of homologous thyroglobulin immune serum revealed any sign of thyroiditis

Animals receiving thyroglobulin antiserum of type A-H showed only a mild degree of thyroiditis after 24 hours Small infiltrations of histiocytes and lymphocytes could be observed But these changes were not significant since animals receiving homologous anti-Freund adjuvants immune serum (antiserum type C) may show such changes (Fig 6) Furthermore, similar histological changes could be observed in the thyroid of animals receiving serum of type A₂L (Table 1)

On the other hand, animals sacrificed 5 or 10 days subsequent to injections of serum A-H in addition to non specific changes revealed aggregation of granulocytes adjacent to the thyroid (see Fig 5) No animal receiving immune serum against Freund adjuvants only (antiserum type C) or against thyroglobulin with low levels of thyroglobulin antibodies (antiserum type A-L) revealed any infiltration of granulocytes in or adjacent to the thyroid gland Liver and kidneys of all animals appeared normal

DISCUSSION

The present study shows that guinea pig anti guinea pig thyroglobulin immune serum may initiate thyroiditis in guinea pigs In parallel with thyroiditis produced by heterologous immune serum (2) the predominant histological feature was a rapid infiltration of granulocytes apparently eosinophils

In contrast to heterologous immune serum, homologous thyroglobulin immune serum did not produce any significant intrafollicular aggregation of granulocytes any remarkable proliferation of histiocytes or a delayed infiltration of lymphocytes Since the quantities of heterologous thyroglobulin antibody employed for injection, as measured by the method of indirect hemagglutination, were 10-70 times that of homologous antibodies, the histologic changes only observed after injections of heterologous immune serum may be explained on a quantitative basis By injection of heterologous thyroglobulin immune serum toxic antigen antibody complexes (at moderate antigen excess, (5)) may be established intrafollicularly, while injections of smaller antibody quantities only produce such complexes in the interfollicular

spaces due to interfollicular presence of small amounts of thyroglobulin.

On the other hand, there may be qualitative differences between heterologous and homologous thyroglobulin immune serum. Although only a single precipitin band could be observed against thyroid tissue in Ouchterlony plates, heterologous thyroglobulin immune serum may contain antibodies to other components of the thyroid which are not present in homologous thyroglobulin immune serum. Finally, since antibodies with the highest affinity to the antigen (7) produced in the same species may be removed from the serum by the thyroid gland of the immunized animal, heterologous immune serum may contain antibodies of higher affinity to the antigen. In this way, a heterologous condition may have features in common with an auto-immune reaction which are not present when auto-antibodies are transferred between animals of the same species.

A mild degree of thyroiditis (+) was observed in animals receiving Freund adjuvants immune serum (antiserum C) or thyroglobulin immune serum with poor anti thyroglobulin response (antiserum A-I). The histologic alterations found in these animals consisted of small infiltrates of mononuclear cells. Since immunization with Freund adjuvants may induce scattered infiltration of mononuclear cells in the thyroid gland (3-8), the present findings indicate that this potential in parallel with other effects of Freund adjuvants (1), may be transferred by serum.

On the other hand, aggregation of granulocytes, presumably of the eosinophilic type, was found only in or adjacent to the thyroid of guinea pigs receiving large quantities of thyroglobulin antibody. This observation strengthens the hypothesis that thyroglobulin serum antibodies may be capable of inducing granulocytic infiltration in or around the thyroid gland.

Apparently the degree and type of granulocytic infiltration are not correlated to injected levels of hemagglutinating antibodies against thyroglobulin. One type of thyroglobulin immune serum (A₁ or B) prepared after 1 month of immunization gave rise to thyroglobulin antibody titers of 1:1000 or 1:500. This type of antiserum initiated an acute interstitial and "capsular" infiltration of granulocytes. The other type of high titered thyroglobulin immune serum (A_{II} thyroglobulin antibody titer of 1:2500) prepared subsequent to booster injections 4 months later initiated a delayed and less pronounced capsular aggregation of granulocytes. "Capsular" infiltration of eosinophils has previously been recognized in rabbit anti-thyroid immunization with thyroid extracts in Freund adjuvants (9).

The two types of antiserum revealed qualitatively reactions in Ouchterlony plates but antiserum A-II manifested higher hemagglutination and precipitation ratio than antiserum A-I. The results of the transfer of antibodies to guinea pig thyroglobulin reactions consist of various types of

immune globulins (4), the explanation behind the lack of correlation between histological alterations and hemagglutinating antibodies may be that different types of immune globulins are involved.

SUMMARY

Thyroiditis in guinea pigs, following intraperitoneal injection of guinea pig anti guinea pig thyroglobulin immune serum, is described. The histological lesions are dominated by infiltration of granulocytes, apparently eosinophils. Although the specificity of the reaction indicates that thyroglobulin antibodies may initiate such changes, the severity of thyroiditis does not correlate with the amounts of thyroglobulin antibody injected as measured by the method of indirect hemagglutination.

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HYPERSEGMENTATION IN NEUTROPHILIC GRANULOCYTES

By

PETER WÄHLBOM

Received 11 VIII 66

Hypersegmentation of the nucleus of granulocytes has long been known to accompany megaloblastic anemia, especially when there is vitamin B₁₂ deficiency. The exact explanation of the phenomenon is not known.

The nuclear formula of neutrophils with respect to segmentation is generally believed to be represented by a bell-shaped curve with a maximum at about three segments (1, 9). There has been some discussion regarding the occurrence of hypersegmentation in other conditions than megaloblastic anemia. Thus, *Edwin et al.* (3) state that they "believe that hypersegmented granulocytes may be found in several other conditions than hypovitaminosis B₁₂". The matter is complicated by the changes in vitamin B₁₂ metabolism which occur in several hematological conditions. These changes have recently been reviewed by *Begemann et al.* (2).

It was thought that a study of the segmentation of neutrophilic granulocytes in a clinical series might contribute to the understanding of the clinical significance of hypersegmentation.

MATERIAL AND METHODS

100 patients were randomly chosen among those who had been subjected to bone marrow examination during 1965. After exclusion of those patients whose B₁₂ or folic acid metabolism was not certainly known there remained 78 patients whose peripheral blood was investigated. The diagnoses in these cases are shown in Table 1. Additionally, 20 healthy control subjects, all nurses or hospital technicians, were studied. Of these healthy controls, 10 were females and 10 were males.

The total number of persons in this study is thus 98. Vitamin B₁₂ deficiency was ruled out or revealed by serum B₁₂ determinations, microbiological determination

immersion with a 10 X ocular and 100 X objective. A differential count was performed after which the neutrophilic granulocytes were injected separately and classed according to their number of segments. When a sufficient number of cells had been examined the number of cells in each class was expressed as a percentage of the total neutrophil count.

TABLE 1

Diagnoses of all Subjects Included in the Study

<i>B₁₂ hypovitaminosis</i>	<i>Infection</i>
7 Addisonian pernicious anaemia	1 Acute upper respiratory infection
1 Intestinal resection syndrome	1 Chronic pyelonephritis
1 Protein losing enteropathy	1 Purulent arthritis
	1 Staphylococcus aureus septicaemia
<i>Iron deficiency</i>	1 Subacute bacterial endocarditis
18 patients	2 Tuberculosis of lymph nodes
<i>Other erythrocytic disorders</i>	<i>Myeloma and macroglobulinaemia</i>
1 Frelthroid aplasia	2 Myelomatosis (Plasmocytoma)
1 Hereditary sphaerocytosis	1 Macroglobulinaemia
5 Polycythaemia vera	
<i>Chronic myelogenous leukaemia</i>	<i>Other patients</i>
1 patient	1 Ankylosing spondylarthritis
<i>Other haematological disorders</i>	2 Cerebral apoplexy
3 Idiopathic thrombocytopenia	1 General arteriosclerosis
1 Allergic purpura	1 Coronary artery failure
1 Eosinophilia of unknown origin	3 Diabetes mellitus
1 Incipient myeloproliferative syndrome	1 Duodenal ulcer
	1 Pleural adhesions
<i>Malignancy</i>	1 Ventricular polyposis
1 Astrocytoma	1 Portal hypertension
1 Bronchial carcinoma	1 Renal arteriosclerosis with hypertension
1 Pulmonary carcinoma	1 Sarcoidosis
1 Ventricular carcinoma	1 Sjogren's syndrome
1 Hepatic carcinoma	1 Spondylarthritis
2 Hodgkin's disease	1 Terminal ileitis
1 Metastatic carcinoma of unknown origin	1 Thyrotoxicosis
	20 Healthy subjects

RESULTS

The results are shown in Tables 2-4. As would be expected, B₁₂ hypovitaminosis forms the group with the most marked tendency to hypersegmentation. In this group, the greatest number of 11 segmented cells is seen, and some cells with 7 segments are also encountered. In several other groups, however, in fact in all except the myeloma group and the healthy controls, occasional cases of hypersegmentation are observed. But 7-segmented cells are seen only in the malignancy group and the infection group, although cases with 6-segmented cells are abundant.

DISCUSSION

The cause of hypersegmentation is unknown. If the phenomenon is interpreted as a sign of vitamin B₁₂ deficiency then the question arises: Do the present results indicate that there was, after all, vitamin B₁₂ deficiency in those patients who had hypersegmented neutrophils but no other detectable signs of such a lack? We know from the investigations of Killander & Larsson (5) that low B₁₂ values may be encoun-

TABLE 2

Numbers of Cells Expressed as Percentages of Total Neutrophil Count

	No. of subjects	Number of nuclear segments*						
		1	2	3	4	5	6	7
B ₁₂ hypovitaminosis	9	0.7 2	0.11 3	8.33 26	32.49 41	5.40 20	0.20 7	0.3 <1
Iron deficiency	15	0.23 7	4.20 10	22.52 40	16.45 33	2.27 10	0.7 3	
Other erythrocytic disorders	7	1.19 7	4.19 10	18.47 35	20.43 33	5.14 12	0.4 2	
Chronic myelogenous leukaemia	1	43	5	20	20	10	2	
Other haematological disorders	6	1.60 13	4.21 12	18.31 31	10.40 33	2.20 10	0.10 3	
Malignancy	8	0.23 12	0.25 16	2.43 31	8.55 29	0.30 10	0.10 3	0.4 <1
Infection	8	1.78 21	3.24 14	16.34 34	6.41 34	0.20 6	0.8 1	0.1 <1
Myeloma and macroglobulinaemia	3	3.7 5	8.41 17	45.50 49	21.37 27	2.7 3		
Other patients	18	0.45 6	0.30 10	20.73 41	4.55 36	0.21 7	0.3 <1	
Healthy subjects	20	1.13 5	11.32 20	34.50 43	16.35 27	1.12 5		

Upper row: Range. Lower row: Mean.

TABLE 3

Number of Subjects with Cells in Each Group

	Total No.	Number of nuclear segments						
		1	2	3	4	5	6	7
B ₁₂ hypovitaminosis	9	7	6	9	9	9	8	2
Iron deficiency	15	16	18	18	18	18	4	
Other erythrocytic disorders	7	7	7	7	7	7	5	
Chronic myelogenous leukaemia	1	1	1	1	1	1	1	
Other haematological disorders	6	6	6	6	6	6	2	
Malignancy	8	7	7	8	8	6	2	1
Infection	8	8	8	8	8	6	2	1
Myeloma and macroglobulinaemia	3	3	3	3	3	3		
Other patients	18	15	18	17	19	14	5	
Healthy subjects	20	20	20	20	20	19		

tered in myelomatosis. In the present study, however, this does not present any problem as the myeloma group did not show cells with more than five nuclear segments. *Hollin & Ross* (7) found high serum B₁₂ values in polycythaemia, whereas *Bjellmann et al.* (2) observed low values. The latter also found low serum B₁₂ values in lymphadenosis and in macroglobulinaemia. All the investigators found high serum B₁₂ values in patients with chronic myelogenous leukaemia. In the present study, the patient with chronic myelogenous leukaemia had some hy-

TABLE 4

Diagnosis of the Patients Who Had Cells with more than Five Nuclear Segments

	6 segments	7 segments
B ₁₂ hypovitaminosis	5 Addisonian p a 1 intestinal resection syndrome	2 Addisonian p a
Iron deficiency	4 patients	
Other erythrocytic disorders	1 erythroid aplasia 1 cong spherocytosis 3 polycythaemia vera	
Chronic myelogenous leukaemia	1 patient	
Other haematological disorders	2 idiopathic thrombocytopenia	
Malignancy	1 bronchial carcinoma 1 hepatic carcinoma	1 hepatic carcinoma
Infection	1 staph septicaemia 1 chronic pyelonephritis	1 chronic pyelonephritis
Other patients	1 ankylosing spondyl arthritis 1 cerebral apoplexy 1 duodenal ulcer 1 renal arteriosclerotic hypertension 1 sarcoidosis	

persegmentation, no abnormality of her B₁₂ metabolism was detected. This matter thus needs further clarification.

It is probable that B₁₂ hypovitaminosis might exist on the cellular level with a normal B₁₂ absorption from the intestine if the B₁₂ requirements are very high, as may be the case in rapid cellular proliferation, such as occurs in leukaemia, polycythaemia and carcinoma. On the other hand, the high serum B₁₂ values observed by several authors in chronic myelogenous leukaemia and in polycythaemia might signify inability of the diseased cells to utilize available B₁₂, owing, for instance, to interference by the disease process with the B₁₂-binding capacity of these cells. Views on the same lines have been expressed by Mollin & Ross (7).

Hypersegmentation should not be regarded as a step in the ageing of granulocytes, for these cells are usually destroyed through nuclear pyknosis without hypersegmentation, as has been shown both *in vivo* and *in vitro* (4, 6). It seems that hypersegmentation is due to a disturbance of the normal metabolic processes in the nucleus. This disturbance may be caused by ineffective utilization of B₁₂, as suggested above, or by some unknown factor independent of vitamin B₁₂.

Schullen (10) regards hypersegmented granulocytes as a more ma-

ture form of the giant metamyelocytes or giant stab cells which are often seen in pernicious anaemia. Of the patients in the present study, only those with Addisonian pernicious anaemia had such giant cells in the bone marrow. Müller (8) studied a patient who had hypersegmented neutrophils during pregnancy in connection with iron deficiency and severe pyelonephritis. He did not see giant metamyelocytes or stab cells in his patient, although the hypersegmented neutrophils were unusually large.

Thus, there is a good deal of support for the assumption of Edwin *et al* (3) that hypersegmented granulocytes would be found in other conditions than hypovitaminosis B₁₂.

Clinically, the matter is less complicated. Neutrophilic granulocytes with hypersegmented nuclei may obviously usually be regarded as a sign of B₁₂ hypovitaminosis. Since hypersegmentation is also seen in other conditions, this phenomenon cannot be interpreted as a sign of vitamin B₁₂ deficiency if other signs are lacking. In conjunction with other signs, however, hypersegmentation corroborates a diagnosis of vitamin B₁₂ deficiency.

SUMMARY

The peripheral blood of 78 patients and 20 healthy subjects was examined with regard to nuclear segmentation of neutrophilic granulocytes. The greatest number of cells with 6 or more segments was found in hypovitaminosis B₁₂. In all other groups except the myeloma group and the controls, however, cases of hypersegmentation were also encountered.

The cause of hypersegmentation is discussed, with special reference to a possible undetectable B₁₂ deficiency. Clinically it is noted that hypersegmentation alone cannot be regarded as a sign of hypovitaminosis B₁₂, but is a useful adjunct to other signs of this deficiency.

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RELAPSING POLYCHONDROSIS

A Histopathological and Histochemical Study of the First Danish Case

By

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Only about 10 cases of relapsing polychondritis are on record. The present case is the first to be observed in Denmark. Its clinical aspects have been published previously (Finn Jensen 1962: 16). Now a post-mortem examination is available, and as only 6 autopsied cases have been published (Altherr & Meyenburg 1936; Harwood 1958; Klagsbrun & Kaltenstein 1958; Parcells, Naham & McNeill 1962; Verity, Larson & Malden 1963 (2 cases)), it would seem of interest to report the pathological and histochemical findings which support the assumption that the disease entails a loss of acid mucopolysaccharides in the cartilage matrix (Golden 1963; Verity, Larson & Malden 1963).

The term relapsing polychondritis was introduced by Pearson, Kline & Newcomer (1960), but the disease has previously been described under a number of different names. In German literature it is usually called Polychondrocythie or Chondromyalgie.

The onset of the disease has occurred at ages ranging from 14 to 61 years. The primary clinical manifestation is recurrent inflammation of cartilaginous tissues, secondarily leading to multiple defects with collapse of the nasal cartilages, the cartilages of the nose and respiratory tract, articular changes, and sometimes inflammatory ocular lesions as well as involvement of the middle ear. The cause of death is usually respiratory insufficiency due to collapse of the air passages or possibly the sequelae thereof. The reported duration of the disease has varied from 1 to 24 years (Haye & Sones 1964).

CASE REPORT

A 45-year-old female

At the time the patient was 45 years of age and the present admission she had an influenza. For 2 days she ran a low grade fever. The auricles which became bluish during the last month before admission had sweats, weight loss, chest pain, and pain in the metatarsophalangeal joints of the ring and little fingers.

She became hoarse, had pain on swallowing and a dry cough. Moreover, she was complaining of both big toes and in the interphalangeal joints of the left hand.



Fig 1

The patient's right external ear at the time of the first admission

When admitted to the Medical Department of St. Lukas Stiftelsen Hospital Copenhagen (case rec 71878) she was on physical examination tired somewhat debilitated anaemic and emaciated Weight 43.8 kg height 158 cm

The external ear (Fig 1) except the lobule was found to be reddish cyanoseal warm swelling with soft thickening of the subcutaneous tissue and partial loss of the scapha. The subcutaneous thickening continued into the external auditory meatus which was almost obliterated. The aurial cartilages were tender and soft making the auricles droop. The bridge of the nose was collapsed at the junction of bone and cartilage (Fig 2). The costal cartilages were tender and swollen at the junction of bone and cartilage reminiscent of a rachitic rosary. There was moderate swelling of the metatarsophalangeal joints of both big toes and a more pronounced swelling of the interphalangeal joints of the left ring and little fingers. The skin overlying the involved joints was rather reddened and the joints were tender.

Laboratory findings ESR 119 mm/hour Hb 10.4 g/100 ml WBC 18,100 differential count showed relative lymphopenia. The sternal bone marrow was the seat of uncharacteristic changes as seen in infections allergic diseases or Hodgkin's disease. Serum iron 0.045 mg/100 ml serum uric acid 5.5 mg/100 ml V R and C N Cus reaction negative Mantoux (10 units) + 8 mm induration. Tb negative on smears and culture. No tumour cells in the sputum. Serum electrophoresis revealed moderately reduced albumin values a slightly reduced α_2 slightly elevated α_1 and γ globulin values FCG Tachycardia but no other abnormalities in particular no signs of myocarditis. Other laboratory tests showed no abnormalities. X rays of toe joints. Mild arthritic changes of the 1st metatars phalangeal joints on both sides. Finger joints. No changes of the interphalangeal joints. Mild hallux valgus.

Biopsy from aurial cartilage (Fig 3) The specimen was covered with normal skin. The underlying thickened oedematous perichondrium showed severe infiltration by lymphocytes and plasma cells and a number of blood filled capillaries. Towards



Fig 2

Patient's profile at the time of the first admission. Note the saddle nose and the thickened somewhat flabby auricle.

acute perichondritis with slight encroachment on the cartilage.

Although the exact diagnosis was in doubt, there was obviously a question of a generalized disease, affecting mostly the cartilage. It was not until later, when Pearson *et al* published their paper (1960), that it was realized that the patient's symptoms could be interpreted as a nosological entity.

Further course. The patient was put on prednisone which she continued to take until she died. The maintenance dose was 5 mg twice daily. On this dosage she was able to keep her full-time job as book keeper, interrupted a few times a year by a week or two of febrile infections in the upper air passages. Only once did she have to be admitted—with pneumonia in 1963.

Terminal stage. In the middle of December 1965 the patient again developed a catarrhal condition, with increasing respiratory embarrassment and difficulty in coughing up the tenacious sputum from the upper air passages. The cough was insufficient owing to the softness of the cartilage. A couple of hours after her admission (case rec 90565) she developed respiratory arrest and shortly after that cardiac arrest. She was immediately given external cardiac massage, and a spontaneous heart beat was re-established in about 3 minutes. Thereupon she had tracheotomy and intubation and manual ventilation. In addition an iv Aramine-P drip was established and she had injections of Celestap (cortisol) and penicillin. A few hours later her condition had become so far stabilized that she could be transferred to the Pispbjerg Hospital Department of Anaesthetics (case rec 41165) for treatment in a respirator. She had to have artificial ventilation and parenteral nutrition. The following signs of severe brain damage. For some days the condition remained stable, but then she developed irreversible hypotension and anuria. Six days after admission without having regained consciousness at any time after the primary cardiac arrest.



Fig 3

Biopsy from the auricle at the first admission. The greatly thickened perichondrium with inflammatory reaction and small vessels occupies almost the entire picture. The ill defined cartilage margin and the relatively intact cartilage may be seen superiorly. Toluidine blue ($\times 500$)

Autopsy (Bispebjerg Hospital, Dept of Path, aut rec R 34/66) 12 hours post mortem. The body of a middle-aged woman whose nutritional state was below medium. Typical saddle nose and deformity of the external ears which were soft to palpation. Tracheostomy. On the arms and legs large, plaque-shaped suggillations and on the left leg a scar after venous cut-down. Joints normal to inspection. The brain weighed 1520 g. There was severe oedema of the cerebrum, and congestive haemorrhages were found in the brain stem. Eyes pale and normal to inspection.

Examination of the viscera showed the abdominal as well as retroperitoneal viscera to be normal apart from mild fatty degeneration of the liver, cholecystitis and cholelithiasis. The adrenals were normal.

Examination of the thoracic organs showed a small patent foramen ovale but otherwise a normal heart. Pleurae and lungs normal apart from pus in the bronchioles. No pneumonic lesions and no atelectases.

The main changes in the respiratory tract were softness and collapse of the epiglottis and laryngeal cartilages, moreover, the trachea had collapsed above the tracheostomy. The mucous membrane was swollen, congested and necrotic in the right side of the trachea. The trachea as well as bronchi were filled with mucopus. When cut, the cartilaginous rings were soft to palpation.

Histological and Histochemical Findings

MATERIAL AND METHODS

Considering that the main object of this investigation was histochemical examination of the cartilage to assess the content of the acid mucopolysaccharides, tissue was removed from the auricular epiglottis trachea and tracheal bronchi. The excelsalls were removed and fixed in Little's fixative for 50 hours while the other specimens were fixed in 10 per cent buffered formalin for 24 hours, dehydrated, cleared in ligroin, embedded in paraffin and cut into sections 5 μ in thickness. In addition normal excelsalls and normal cartilage from auricular epiglottis trachea and bronchi were used as control. The remaining biopsy material was regrettedly too scanty for histochemical examination.

The full range staining methods were employed:

(1) Haematoxylin-eosin (2) van Gieson-Hansen (3) Wilt's reticulum stain (4) elastic tissue staining by Verhoeff's method (modified) (5) Alcian blue (AB) by the Day and Eskeland methods (6) the PAS reaction after McManis (PAS) (7) Best's carmine (8) PAS full range diastase hydrolysis at 37°C for 60 minutes (9) colloidal iron stain (10) by the method of Barka (10) colloidal iron + PAS (11) PAS (11) 0.5 per cent aqueous toluidine blue (TBL) for 20 min (12) 0.1 per cent azure A (AA) for 10 min at pH 2 to 5 (13) sulphation with sulphuric acid after Lewis & Griffin followed by the Kramer & Windrum technique for metachromaticity (14) aldehyde fuchsin Alcian blue (AF-AB) after Spicer & Meyer (15) Alcian blue safranin O (AB-S) (16) N,N-dimethyl-m-phenylene diamine + N,N-dimethyl-*para*-phenylenediamine-Alcian blue (D-AB) by the method of Ley & Spicer (18 hours) and (17) mercury bromphenol blue staining (MBH B).

RESULTS

Normal Cartilage

All control cartilages showed uniform reactions. However, staining of reticulin and elastic fibres was more pronounced in the elastic cartilage of the auricle and epiglottis than in hyaline cartilage. PAS positive reactions were found particularly in the cartilage matrix but also more faint in the cytoplasm of the chondrocytes. The PAS reaction was unchanged after diastase digestion although Best's carmine showed quite pronounced staining of elastic as well as hyaline cartilage. Alcian blue gave a positive reaction only in chondrocytes, faint staining of the perichondrium but deep staining of the lacunar rim. The same applied to colloidal iron which, however, stained the matrix faintly. Combined colloidal iron and PAS gave a blue colour to chondrocytes as well as to the lacunar rim and magenta to the matrix.

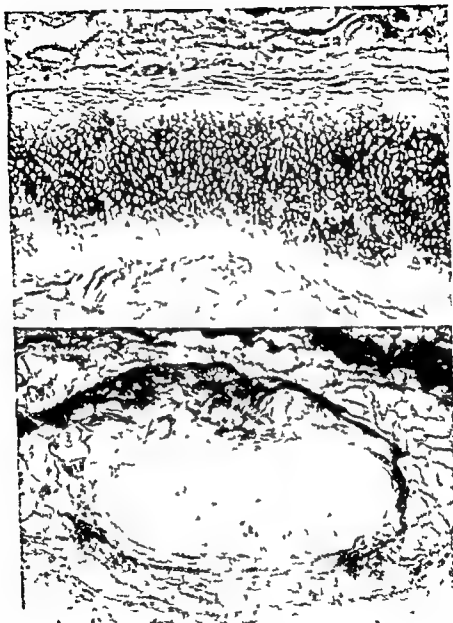
Aldehyde fuchsin Alcian blue Alcian blue safranin and iron diamine Alcian blue showed deep staining of the matrix by aldehyde fuchsin and safranin O and black staining by iron diamine. Toluidine blue as well as azure A gave pronounced metachromasy which with toluidine blue comprised the chondrocyte layer of the perichondrium but never reached the external layer. At pH 2 there was still a faint metachromatic reaction with azure A increasing to a maximum around pH 4. Following sulphation there was a definite increase in the staining intensity.

Mercury bromphenol blue showed faint staining of the matrix and the surrounding tissue also showed a faint reaction.



Fig. 5

Section from the abnormal cartilage of the trachea. Note the pronounced defects in the cartilage, replaced by connective tissue. In the defects there is still, especially in the lower picture, infiltration by inflammatory cells.
Colloidal iron-PAS (top $\times 200$, bottom $\times 500$)



Fig

Fluorescent tissue staining of normal (top) and pathological hyaline cartilage (bottom). The latter has completely transformed into a connective tissue plate without any fibrillar structure. Stained with Verhoeff ($\times 200$)

TABLE 1
Staining Reaction of Normal and Pathological Cartilage

Staining method	Control	Auricle	Epiglottis	Trachea	Bronchi
Wilder	+++	+	+	—	—
Verhoeff (mod)	+++	+	+	—	—
PAS	+++	+++	+++	+++	+++
Best's carmine	++	++	+	+	+
PAS post diastase	+++	+++	+++	+++	+++
AB	+++*	+	+	—	—
CI	+++*	+	+	(+)	(+)
CI - PAS	+++* § +++	(+) +++	— +++	— +++	— +++
AF AB	+++§ +	+	+	(+) +	(+) +
AB - S	+§ +++	+	+	(+) —	(+) —
DI - AB	+++§ +	(+) +	(+) +	— (+)	— (+)
TBI	+++	++	+	+	+
AA pH2	(+)	—	—	—	—
AA pH3	++	—	—	—	—
AA pH4	+++	+	+	+	+
AA pH5	+++	++	++	+++	+
Metachromasia post sulphation	+++	+	+	+	+
MBPB	+	+	+	+	+

Symbols - Staining method symbols. See material and methods

— = no staining

(+) → +++ = degree of staining

* Reactivity only in chondrocytes, lacunar rims and perichondrium

† Reactivity present in the surrounding tissue and the regenerative connective tissue

§ Upper row Reactivity of the first dye in the sequence

Lower row Reactivity of the second dye in the sequence

Normal eyeballs showed a positive colloidal iron and Alcian blue staining in the layer of rods and cones as well as pronounced β metachromasia of the corneal stroma. These methods caused less intense staining of the sclera, but a marked reaction in the vitreous body.

Cartilage from the Autopsy Material

In general it may be said that the changes of the cartilage and cartilage remnants were of a chronic, fibrous nature unlike the exudative, inflammatory changes in the early stage of the disease seen in the biopsy specimen. All the cartilage of the autopsy material was char-

acterized by severe changes. The margin was everywhere eroded. The resulting defects were replaced by collagenous connective tissue, in most sites without infiltration by inflammatory cells. In the trachea however there were remnants of inflammatory exudate in the major defects (Fig. 4). In several sites, especially in the bronchi, the cartilaginous tissue had entirely disappeared being replaced by a connective-tissue plate whose outlines corresponded to those of the original cartilage (Fig. 5).

In the residual cartilage islands the number of chondrocytes had greatly decreased and there were several empty lacunae. Furthermore the fibrillar structure of the matrix demonstrated in normal cartilage by reticulum and elastic tissue stains had greatly decreased or been lost (Fig. 5).

Most interest attaches to the various methods for demonstrating mucopolysaccharides.

The metachromatic reactions were far weaker in the pathological cartilage but positive in the reparative connective tissue. However much depended on the pH as no staining was obtained at the more acid reactions. In the normal cartilage β metachromatism was still present in a highly acid medium. There was no increase following sulfation. A characteristic finding of importance to pathogenetic considerations was the pronounced β metachromatism in the tissues surrounding the diseased cartilage, a reaction which was often as pronounced as in normal cartilage. This strong metachromatic reaction was in marked contrast to the weaker reaction in the reparative connective tissue and to the sharp demarcation of the metachromatic reaction in normal cartilage (Fig. 6).

As illustrated in Table I the other staining reactions were also greatly reduced in the pathological cartilage. However colloidal iron as well as Alcian blue gave fairly pronounced diffuse staining of the reparative connective tissue and of the surrounding normal tissue. This is a considerable difference from normal cartilage in which these dyes stained mainly chondrocytes, lacunar rims and perichondrium. The reactivity was also far greater than in normal connective tissue. Aldehyde fuchsin, Alcian blue and Alcian blue-safranin also showed a blue staining of tracheal and bronchial cartilages while there was a faint aldehyde fuchsin and safranin staining of the uveolar and epiglottic cartilage. Iron diamine staining was negative in the pathological cartilage but there was a positive reaction at the site of bronchial glands. An extremely characteristic finding was the persistent PAS positivity in the residual cartilage matrix while PAS positivity was not observed in the surrounding tissue or in the reparative connective tissue. PAS staining was unchanged after diastase hydrolysis.

Examination of the eyes revealed slight degeneration of the elastic fibres in the conjunctiva and in the left eye a small retinal detachment in the macular area. The histochemical reactions on the other hand showed no difference from the control eyes.



Fig 6

Metachromatic reaction in normal and pathological cartilage. Note the sharp demarcation between the cartilage and surrounding tissue in the normal cartilage (top) and the faint staining in the central areas of the diseased tissue (bottom). On the other hand the bottom picture shows intense metachromatic staining of the surrounding tissue. Azure A at pH 4 ($\times 200$)

DISCUSSION

The clinical course and the pathological findings correspond accurately to previous cases published as relating polychondritis. *Verity, Larson & Madden* (1963) as well as *Kaye & Sones* (1964) have listed the affected organs. In practically all cases the cartilage of the larynx, nose and respiratory tract has been involved, but cartilage lesions may be observed in all parts of the body including articular cartilage.

The eyeball and the heart though non cartilaginous are stringently enough often involved. This is possibly due to the high content of acid mucopolysaccharides of the eyeball and of the cardiac valves. The ocular changes manifest themselves as iridocyclitis or episcleritis. *Kaye & Sones* (1964) found the eyeballs to be involved in 24 out of 75 patients. The mural manifestations have been described in detail by *Bueker & Ferguson* (1965).

Examination of the eyeballs from the present patient showed no pathological or histological changes which could be ascribed to polychondritis. And indeed this was not to be expected as there had been no clinical ocular symptoms.

As in most previous cases the cause of death was increasing respiratory failure due to collapse of the respiratory tract. It is typical that nearly all the patients had been tracheostomized immediately before death.

Histological examination shows a characteristic development from exudative-inflammatory mainly perichondrial changes with initially intact cartilage to a chronic fibrous stage. At this stage marginal erosion of the cartilage is seen with breakdown of hyaline and chondrocytes, the defects being filled with connective tissue. At first this connective tissue is the site of moderate inflammatory reaction. Later it is fibrotic. The histological changes observed in the biopsy specimen and at autopsy reflect this sequence of development.

The pathogenesis is elucidated by the histochemical examination of the cartilage.

The composition of the cartilage matrix has not yet been fully established (*Handbuch der Histochemie* 1964). It has been known for a long time that the matrix contains large quantities of chondroitin sulphuric acid. According to recent investigations (*Meyer et al* 1956) three quarters of the mucopolysaccharides are made up of chondroitin sulphate A while chondroitin sulphate C is present in smaller quantities. There is not yet agreement as to whether chondroitin sulphate B is present. At any rate it is not mentioned as a component of cartilage by *Thompson & Hunt* (1966). Other components isolated from the ground substance are keratosulphate a sulphated aminopolysaccharide (*Meyer et al* 1958) and chondroitin a non sulphated mucopolysaccharide (*Davidson & Meyer* 1954). Chondroitin sulphate A and keratosulphate are also present in large quantities in the cornea (*Meyer et al* 1955).

No histochemical method is absolutely specific for the demonstration of acid mucopolysaccharides in general or for their individual subgroups in particular, but a combination of several methods may give an impression of the localization of these substances in the tissues. The reliability is increased if autoradiography with $S^{35}O_4$ is used for comparison. However, there is not agreement concerning the interpretation of the various reactions.

According to Spicer (1963) the purple and the red colour seen in the cartilage matrix with an aldehyde fuchsin-Alcian blue and an Alcian-blue-safranin sequence indicates a content of highly acid sulphated mucopolysaccharides. In the same way, Lev & Spicer (1965) state that sulphated mucosubstances stain black with iron diamine stains. The sulphated mucosubstances give pronounced β -metachromasia which, however, is given also by acid, non-sulphated mucopolysaccharides (Wislocki *et al* 1947, Spicer 1963). All sulphated acid mucopolysaccharides do not, however, show metachromasia (Spicer *et al* 1961). According to Spicer (1963) certain, non-sulphated acid mucopolysaccharides show orthochromasia.

A survey on the various sulphated acid mucopolysaccharides and their staining reactions has been given by Spicer (1960). According to Spicer & Meyer (1960) an aldehyde fuchsin-Alcian blue sequence is applicable for differentiating between sulphate and carboxyl groups.

Polysaccharides containing mainly carboxyl groups stain with colloidal iron and Alcian blue (Mowry 1963), while neutral mucopolysaccharides are PAS-positive and diastase resistant.

According to Table 1, highly acid sulphated mucopolysaccharides and neutral mucopolysaccharides appear to be present, especially in the ground substance, while chondrocytes, the perichondrium and the perichondrium contain particularly non sulphated acid and neutral mucopolysaccharides. A large part of the PAS-positive substances are presumably mucoproteins.

Table 1 shows, moreover, that the pathological changes increase downwards in the respiratory tract. The elastic cartilage in the auricle and epiglottis is involved to a lesser extent and the hyaline cartilage of the bronchi most severely. In the most cranial part of the trachea the hyaline cartilage is best preserved, though greatly eroded. These cartilage remnants still show PAS positivity while reactions for acid mucopolysaccharides are negative. In the surrounding connective tissue, however, there is intense staining with Alcian blue and colloidal iron but slight staining with aldehyde fuchsin. These findings presumably indicate leaking of acid mucopolysaccharides from cartilage and cartilage remnants to the surrounding tissues.

Two pathogenetic hypotheses have been suggested *viz*:

- (1) That the perichondrial inflammation is primary and the destruction of cartilage secondary.

- (2) that the disease process affects the cartilage primarily, the perichondrial reaction being secondary.

These hypotheses have been thoroughly discussed by Verity *et al* (1963). They conclude that most findings indicate that changes of the cartilage matrix with loss of acid mucopolysaccharides are primary. In this respect the finding of an increased urinary excretion of acid mucopolysaccharides is of interest (Golden 1963; Rucker & Ferguson 1963). This increased excretion is seen especially during relapses. In quiescent phases of the disease the urinary excretion of acid mucopolysaccharides has been found to be normal (Kaye & Sones 1964).

Experiments have shown that intravenous injection of the proteolytic enzyme pepsin into rabbits induces cartilage changes of a nature similar to that in human polycondritis (Thomas 1956; Spicer & Bryant 1957; Westerlund 1961). However, this has not led to ocular changes, only cartilage changes. Whether enzymatic defects play a role in the human disease still remains unclarified.

Since similar cartilaginous changes, though of lesser severity, are observed in systemic lupus erythematosus, Hashimoto's thyroiditis, rheumatoid arthritis, and Sjögren's syndrome (Kaye & Sones 1964), and since corticosteroid therapy has a certain effect, some authors have classified the disease as a collagen disease, while others consider it to be an autoimmune disease.

The present study supports the hypothesis that acid mucopolysaccharides disappear from the cartilage matrix gradually as the disease progresses, but we are not prepared to say whether this is the primary defect or a secondary phenomenon. More advanced histochemical procedures (enzyme histochemistry, immune histochemistry, etc.) may possibly throw further light on the aetiology and pathogenesis of this disease.

SUMMARY

The first Danish case of relapsing polycondritis previously described clinically is now reported from the pathological and histochemical aspects.

The patient died in a way typical of the published cases of respiratory failure following collapse of the upper air passages.

Histological examination revealed destruction of the hyaline cartilage increasing towards the distal end of the trichotracheal tree. The elastic cartilage of the nodule and epiglottis appeared to be less severely involved.

Histochemical staining methods indicated a loss of acid mucopolysaccharides from the cartilage matrix. However, the present study did not afford a basis for concluding whether this is the primary or secondary to the early occurrence of polycondritis.

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DIFFERENTIATION OF LARGE VACUOLATED NEOPLASTIC CELLS IN EPIDERMIS BY MEANS OF PAS STAINING

By

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Received 25 VI 1967

Distinction between Paget cells and neoplastic cells from melanoma penetrating the epidermis is described by Allen & Spitz (1953) may sometimes cause difficulties to the pathologist, as pointed out by Lever (1961). The difficulties in distinguishing Paget's disease from early melanoma have been demonstrated by Culbertson & Horn (1956) who found that Paget cells may contain melanin. It was reported by Fisher & Beyer (1959) that periodic acid-Schiff positive material could be identified in the cytoplasm of many of the large intraepidermal cells of Paget's disease in all lesions investigated while this was not the case of melanomas. In the latter lesion cells containing melanin when stained by this technique frequently showed a content of rust coloured cytoplasmatic material. In contrast to the slight PAS positive material which may be found in the surrounding squamous cells the PAS positive material in Paget cells does not disappear after diastase digestion. From these studies it seems as if the PAS staining may provide means of distinguishing between the mentioned lesions. On the other hand it appears that such an assumption would build on 8 cases of melanomas and 3 cases of Paget's disease. The author has therefore attempted to test the differentiation by means of PAS staining on a larger material.

MATERIAL AND METHODS

The present material consists of 30 cases of Paget's disease and 25 malignant melanomas. Sections were prepared from formalin fixed blocks and stained by the periodic acid-Schiff reaction haematoxylin-silver and alcian blue staining.

RESULTS

The results of the tinctorial reactions are listed in Table 1. It is seen that no intraepidermal cells of malignant melanoma contained PAS positive material in contrast to Paget's disease among which 9 cases

out of 36 showed intracutaneous tumour cells containing small granules of distinct PAS positive material. One case appeared to demonstrate several large neoplastic cells in the epithelium showing a considerable amount of PAS positive material. It is further noted that none of the sections from Piglet's disease as well as from melanomas contained distinct Alcian blue positive material in the cytoplasm of the large intracutaneous tumour cells.

TABLE I

Staining Reactions of the Cytoplasm of Large Intracutaneous Neoplastic Cells in Piglet's Disease and Malignant Melanomas

No. of cases		PAS Reaction		Alcian Blue Reaction	
		Positive/Negative		Positive/Negative	
36	Piglet's disease	9	27	0	36
23	Melanomas	0	23	0	23

DISCUSSION

The utilization of histochemical procedures may prove valuable in tumour differentiation as shown by *Hurdstone* (1961). The differentiation by means of PAS-positive material in the large vacuolized intracutaneous cells of Piglet's disease have, however, in this material been of less value as 75 per cent of the cases studied appeared to have no PAS positive material in the large optically clear cells situated in the epithelium. This is in contrast to the material presented by *Fisher & Hoyer* (1959) who found PAS positive material in all lesions investigated. None of the melanomas appeared to contain PAS positive substance but often a rusty coloured material were found in cells containing melanin which correspond with *Fisher & Hoyer* (1959) and *Pearse* (1961). None of the six neoplastic lesions showed a positive alcianblue staining.

SUMMARY

Thirty-six cases of Piglet's disease and 23 malignant melanomas were investigated by means of PAS and alcian blue staining techniques as to their contents of PAS positive material in the large vacuolized intracutaneous cells found in the neoplasms mentioned. Only 9 cases of Piglet's disease out of 36 were found to possess PAS positive material in the large intracutaneous cells while none of the melanomas gave positive reaction. The alcian blue staining was negative in all cases investigated. The PAS staining may thus be considered a useful means in differentiating between certain cases of Piglet's disease and malignant melanomas. A negative reaction however does not exclude the presence of Piglet's disease.

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HUMORAL IMMUNITY DURING THE INDUCTION OF EXPERIMENTAL AMYLOIDOSIS

By

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That immunological phenomena very probably play an important role in the development of experimental amyloidosis is generally accepted. It is well known that a wide range of materials are effective in inducing amyloidosis in many species, especially if such materials are capable of acting as antigens themselves or indirectly if they induce suppuration in the treated organism. In histological investigations on amyloidosis in mice subjected to hyperimmunization with casein it appeared that the formation of amyloid in the tissues depended on a biphasic mechanism: an initial pre-amyloid phase with proliferation of reticuloendothelial pyroninophilic cells and plasma cells and rise in the serum gamma globulin, a second amyloid phase with decreasing pyroninophilicity and the appearance in rapid succession of PAS-positive reticuloendothelial cells in the border zone of new formed amyloid deposits (Teilmann 1956, 1961a). This cytochemically well defined transition from a pre-amyloid to an amyloid phase was believed to be the result of an exhaustion leading to a functional break down of the immune process responsible for the initial pyroninophilic phase, the exhaustion being the consequence of a prolonged and heavy antigenic stimulation. These studies showed a definite functional relationship between reticular cells and amyloid, but the type of immune reaction apparently taking part in the pathogenesis of amyloidosis remains ill defined. Based on the assumption that this alleged immune dysfunction triggering the amyloid phase might reflect itself in a general disturbance in one or more of the immune mechanisms, we decided to investigate the effect of an amyloid inducing regimen on the humoral host defence mechanism: phagocytic activity, delayed (cellular) hypersensitivity, and the capacity to form circulating antibody. In functional studies of the phagocytic properties of the R.E.S. during the development of casein induced amyloidosis in mice phagocytosis was found to vary with the

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phasic development of the disease the pyroninophilic, preamyloid phase was accompanied by a steady increase in phagocytosis of colloidal carbon, while the second, amyloid phase coincided with a decrease in phagocytic activity (Ranlov 1966b). Another experiment showed a significant delay in skin homograft rejection in casein-treated mice during the amyloid phase (Ranlov & Jensen 1966).

The present experiment is an attempt, under similar conditions, to determine the capacity to form circulating antibodies towards another antigen, ferritin, in groups of mice primarily treated with casein for different lengths of time.

MATERIAL AND METHODS

72 closely inbred C3H mice of equal sex distribution were used. They were from 10 to 15 weeks old at the beginning of the experiment. They were fed on ordinary food pellets and water except during the casein treatment when they were fed on oats. After randomization they were divided as follows:

- 12 control mice
- 12 mice treated with casein for 1 week (6 injections)
- 12 mice treated with casein for 2 weeks (12 injections)
- 12 mice treated with casein for 4 weeks (24 injections)
- 12 mice treated with casein for 6 weeks (36 injections) and
- 12 mice treated with casein for 8 weeks (48 injections)

Pretreatment 0.5 ml of a 5 per cent solution of casein in 0.25 per cent sodium hydroxide was injected subcutaneously in different sites of the back daily 6 times a week.

Ferritin immunization The day following the last casein injection (in the untreated controls on the first day of the experiment) each animal received an intraperitoneal injection of 0.1 mg horse spleen ferritin (Fluka A.G.) in 0.5 ml sterile saline mixed with an equal volume of Freund's complete adjuvant (Difco). After 7 days each animal was given an additional dose of 0.1 mg ferritin in 0.5 ml sterile saline intraperitoneally. 6 days after the booster injection the animals were killed by heart puncture under ether anaesthesia. The blood was allowed to clot for half an hour at 37°C, centrifuged and serum was stored at -20°C.

Pathology Lung, thymus, liver, spleen, kidney, adrenal, ilium and mesenteric lymph node were fixed overnight in 10 per cent neutral formalin, paraffin embedded and sections were cut 5 microns thick. They were stained with methyl green pyronin and with the PAS technique.

Antibody titration The titre of antiferritin antibody was estimated in serial dilutions of each mouse serum beginning at 1:20. A passive haemagglutination technique with formalinized sheep red blood cells was used, all sera being analysed in the same procedure on the same day.

Sheep red blood cells were washed 6 times in cool saline and made up to an 8 per cent suspension to which was added an equal volume of a 3 per cent solution of formaldehyde in saline. This mixture was left overnight at 37°C, washed 3 times and resuspended in saline. A 10 per cent suspension was stored at 4°C. 2 ml of this suspension was washed once in 40 ml phosphate buffered saline (PBS) (phosphate buffer 0.05 M, pH 7.0 and normal saline 1:4) and resuspended in 40 ml PBS mixture of 4 ml freshly prepared tannic acid (0.1 per cent) and 56 ml PBS was added after which the mixture was left at 37°C for 15 minutes, centrifuged in the cold for 5 minutes at 4000 rpm. The wash was repeated with 100 ml of PBS. The mixture was resuspended in 30 ml of PBS followed by the addition of a freshly prepared solution of 10 mg of ferritin in 50 ml PBS. The sensitization was carried out at 37°C for 30 minutes. After centrifugation resuspension in 100 ml PBS and centrifugation the sensitized red cells were washed in 100 ml PBS containing 1 per cent normal rabbit serum the latter previously being absorbed twice with formalinized sheep red blood cells. Finally the sensitized sheep red blood cells were

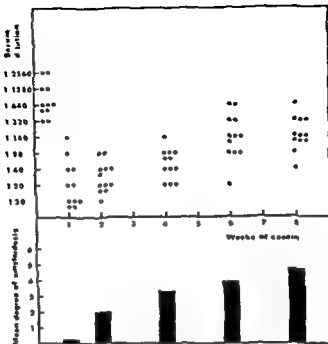


Fig. 1

Serum anti-ferritin antitoxin titres (1:1) and mean degrees of amyloidosis (bottom) in groups of mice pretreated with casein for different lengths of time. The titres at the time "0" represent non-casein treated controls.

made up and adjusted as a 10 per cent suspension in PBS with 1.5 per cent albumin normal rabbit serum.

0.2 ml of the serum dilution was mixed with 0.1 ml of a 2 per cent suspension of sensitized sheep red blood cells and left overnight at 4°C. The reactions were read as + + + + +, + + +, and + + + - respectively. The titre was expressed as the dilution in the last well showing positive haemagglutination (non-ferritin negative).

As negative controls were used normal rabbit serum and PBS. As positive controls a rabbit serum of known titre.

RESULTS

The main results of the experiment are graphically outlined in Fig. 1. The spleen being the primary and most constant site of amyloid formation in our system (Christensen 1957) sections of this organ were chosen for the purpose of estimating quantitatively the degree of amyloidosis as described by Christensen & Hjort (1959). However it should be emphasized that the mean degrees of amyloidosis for each of the groups presented in Fig. 1 do not truly represent the amounts of amyloid present in the spleens after termination of the casein treatment but represent the amounts of amyloid present in the spleens after additional 2 weeks of ferritin immunization. According to experience amyloid formation stops with the termination of casein administration.

and reabsorption does not take place (*Christensen 1963*). On the other hand, injections of Freund's adjuvant alone or in combination with other antigens may cause amyloidosis (*Christensen 1962*). In the present material the combined treatment with ferritin and Freund's adjuvant probably caused the minute amounts of amyloid observed in one of the 12 control mice. Thus small amounts of amyloid have probably been added to every group of animals as a side effect of the ferritin immunization. However, as it does not influence the conclusions of the present experiment no attempts of correction have been made. With this modification the variations in mean degrees of amyloidosis between the various groups corresponded well to those observed under similar conditions in earlier experiments (*Rantlov 1966b*, *Rantlov & Jensen 1966*). Significant amounts of splenic amyloid were not seen until 2-3 weeks after the initiation of the casein treatment. After this the spleen amyloidosis gradually increased, concurrent with increasing amyloid infiltration of the periportal areas of the liver, the renal glomeruli, the lungs and, finally, the inner layer of the adrenal cortex.

In Fig. 1 the anti-ferritin antibody titre for each single animal is plotted against the length of the casein treatment. Among the controls the lowest titre was 320, the majority of controls being between 640 and 1280. In the group receiving casein for one week prior to ferritin immunization the mean antibody titre had fallen to a value between 20 and 40, 4 animals showing titres below 20. After 2 weeks of casein a slight increase occurred, the mean titre still being between 20 and 40 but only one animal showed a titre lower than 20. After 4, 6 and 8 weeks of casein a steady increase towards control values of anti-ferritin antibody titres was evident, the titre at 8 weeks being between 160 and 320. No relation between antibody titre and degree of amyloidosis in individual animals was noted.

DISCUSSION

A possible participation of immunoglobulins and circulating antibodies in the pathogenesis of amyloidosis has been the subject of much speculation. *Toeschicke (1927)* regarded amyloid as a precipitate of antigen-antibody complexes and modified versions of this view have later been put forward (*Letterer 1934*, *Latvalahti 1953*). However, investigations by *Vazquez et al (1957)* and *Calkins et al (1960)* have not been able to confirm such hypotheses. Further, no correlation between the amount of serum anti-casein antibody and degree of amyloidosis has been shown to exist in rabbits (*Giles & Calkins 1958*) or in mice (*Rantlov & Ebbesen 1965*). Most investigations of the serum proteins during the induction of experimental amyloidosis have shown a steady increase in the gamma fraction regardless of the amount of amyloid formed as a result of the amyloidogenic treatment (*Giles & Calkins 1958*, *Clausen 1960*, *Christensen & Rask-Nielsen 1962*, *Rantlov 1966a*). In the present

experiment the capacity to form circulating antibodies returned to wards normal with increasingly severe amyloidosis. Compared with the observations of amyloidosis developing in agammaglobulinemic individuals showing the antibody deficiency syndrome (*Gras et al* 1954, *Teilum* 1964b, *Squire* 1965) the above mentioned and the present observations seem to argue against any definite relationship between humoral immune mechanisms and mechanisms involved in amyloid formation.

Different cells or cellular mechanisms seem to be concerned in the synthesis of circulating antibody and in cellular hypersensitivity reactions. Hypo- and agammaglobulinemic individuals usually respond well with delayed hypersensitivity reactions in spite of an almost total lack of plasma cells and ability to produce circulating antibodies (*Porter* 1955, *Kelly et al* 1958, *Arenberg & Leskowitz* 1963). On the other hand patients with reticulososes such as sarcoidosis or Hodgkin's disease usually produce circulating antibodies normally while frequently failing to develop delayed hypersensitivity reactions (*Rosenberg* 1951, *Sokol & Primultrios* 1961).

In contrast to the findings in the present experiment of an increasing ability to form circulating antibodies with increasing amyloidosis is the decrease in phagocytic capacity with increasing amyloidosis found in rabbits (*Sherrington et al* 1965) and in mice (*Ratnow* 1966b). This may indicate a functional relationship between amyloid producing mechanisms and reticuloendothelial cells corresponding to the earlier described relationship (*Teilum* 1956). In the same way the fact that homograft rejection is inhibited with increasing amyloidosis (*Ratnow & Jensen* 1966) seems suggestive of a participation of immune reactions akin to the cellular delayed type in the pathogenesis of amyloidosis. As it is well known that delayed hypersensitivity reactions such as allergic encephalomyelitis (*Wetrom & Walzman* 1962), experimental allergic thyroiditis (*Helix-Duriez & Walzman* 1961) and experimental allergic nephritis (*Hess et al* 1962) may be transferred to normal recipients with spleen or lymph node cells it is of interest to note that experimental amyloidosis in the same way has been transferred to normal syngeneic recipients by means of injections of spleen cells from casein treated donor mice (*Werdelin & Ratnow* 1966).

In contrast to the almost no real antibody response after 6 and 8 weeks of casein treatment is the marked depression of the same response observed in mice which have received casein for only one or a few weeks prior to the ferritin stimulation (Fig. 1). The reason for this temporary suppression is not obvious but it seems most likely to be the result of an antigenic competition between casein and ferritin.

Antigenic competition is generally accepted as a real immunologic phenomenon which under appropriate conditions can be demonstrated with many different types of antigens. Among others *Weller et al* (1961) described severe mutual reactions in the antibody responses to 5 different bacterial and protein antigens when

injected simultaneously into the same mouse. They further described the delay in skin homograft rejection after large doses of other antigens presumably unrelated to the transplantatic antigen. It is possible that the delay in skin homograft rejection in guinea pigs may be due to the simultaneous injection of bacteria (Rapaport 1961). Recently injected with antigen mainly acting in reducing the primary response.

In chickens it has been shown that the simultaneous injection of bovine serum albumin (BSA) and human gamma globulin (HGG) did not result in significant reductions in either antibody response. However, if the injection of the same amount of HGG was delayed for 24 hours after the BSA injection the antibody response to the latter antigen was significantly reduced (Abramoff et al 1961). In guinea pigs a primary stimulus of diphtheria toxoid suppressed the response to a course of two injections of tetanus toxoid when the first of these was given at times ranging from 3 to 21 days later, the "competitive" effect being maximum at day 7 (Barr & Llewellyn-Jones 1955). Thus it seems that the timing of the different antigenic stimuli is critical and specific for the various experimental systems employed.

Very probably the dosage, too, is critical. Adler (1964) showed that small doses of bovine gamma globulin given together with ferritin enhanced the antibody titre to ferritin while larger doses of bovine gamma globulin depressed it. In this connection it should be emphasized that the single and the accumulated dosage of casein administered to the various groups in the present experiment was considerably larger than were the amounts of "competing" antigen employed in other experiments reported in the literature, in the present material approximately 1 000 mg per kg body weight (daily) as compared to 20-50 mg in single or a few doses (Abramoff et al 1961; Rowinski et al 1966). That the inhibition of the response to a second unrelated antigen may be proportional to the amount of "competing" antigen administered has been regarded as supporting a concept of antigenic competition being a competition for antibody-producing cells or their precursors (Adler 1964). Such a "clonal" concept may easily explain the markedly depressed anti-ferritin antibody response in the present material after one or two weeks pretreatment with casein as a "crowding out" effect—most of the available antibody producing cells being engaged in coping with the casein. However, it offers no explanation of the rising anti-ferritin antibody titre following larger amounts of casein. The reason for this discrepancy is not apparent, it might be attributed to a slowly developing state of tolerance towards casein, or it may be caused by a slowly developing compensatory increase in the number of non-committed clones.

In summarizing the results of the present and earlier reported experiments on the functions of the immune mechanisms during long-standing antigenic stimulation leading to the development of amyloidosis in mice it may be stated that 1) the primary, pyroninophilic preamyloid phase is characterized by a) a rapid increase in phagocytic capacity (Rantow 1966b), a probably normal cellular hypersensitivity reactivity (Rantow & Jensen 1966) and c) a rapid decrease in the capacity to form humoral antibody against other antigens. 2) The second amyloid phase is characterized by a) a steady and marked decrease in phagocytic capacity (Rantow 1966b), b) a significantly reduced cellular hypersensitivity reactivity (Rantow & Jensen 1966) and c) a normalization of the capacity to form humoral antibodies against other antigens in spite of an increasingly severe amyloidosis. These findings seem to imply a participation of reticuloendothelial cells and immune mechanisms akin to the delayed or cellular type in the pathogenesis of experimental amyloidosis.

A number of other investigations support the assumption of a connection between reticular tissues, cellular immune reactivity and amy-

lousis. Graft rejection and graft versus host reactions are both believed to rely on cellular immune mechanisms and both are mediated through large pyroninophilic cells (Gowans 1962) indistinguishable from the large pyroninophilic reticular cells constantly seen dominating the tissues during the premyeloid phase. Small lymphocytes believed to be active in cellular immune reactions are deficient in the peripheral blood during the myeloid phase (Fatalelli 1953 Christensen & Raul Nielsen 1962). In this connection it is noteworthy that the large pyroninophilic cells of Gowans have been shown to be the result of transformation of small lymphocytes. The fact that immune reactivity of the cellular (or delayed) type may be transferred with spleen or lymph node cells while humoral immune reactivity can not correspond to the observation that amyloidosis can be produced by transplantation of spleen cells from casein treated mice to normal syngeneic recipients (Werdelin & Rantow 1966) lends further support to the assumption of cellular immune reactivity playing a role in the pathogenesis of amyloidosis.

SUMMARY

In continuation of earlier investigations of the influence of an amyloid inducing regimen on phagocytosis and delayed hypersensitivity reactivity in mice an experiment under similar conditions has been carried out in casein treated mice in order to define their capacity to form humoral antibodies against another antigen ferritin at various stages of the amyloid induction.

It was found that mice treated for one week with casein showed a marked decrease in serum anti ferritin antibody the titre being lowest at this stage. After 2 weeks of casein pretreatment the titre began to rise. With increasingly severe amyloidosis after 4, 6 and 8 weeks of casein a steady increase in the capacity to form anti ferritin antibody was evident the response after 8 weeks approaching control values. This temporary suppression of humoral antibody response is discussed as a phenomenon of antigenic competition.

Based on earlier observations of decreasing phagocytic capacity and delayed homograft rejection with increasing amyloidosis in mice in contrast to the normalizing humoral antibody response found in the present material a participation of reticuloendothelial cells and immune mechanisms akin to the delayed cellular type in the pathogenesis of amyloidosis is suggested.

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REACTION OF ANTINUCLEAR FACTORS WITH POLYMORPHONUCLEAR GRANULOCYTES

2 On the Inaccessibility of Intact Leucocytes to Granulocyte-Specific ANF of High Molecular Weight

By

PREBEN ELLING

Received 26 VIII 66

Antinuclear factors (ANF) which reacted selectively with granulocytes in human blood smears, but not with other mesenchymal or epithelial cell nuclei, have been found in some sera as the only ANF detectable by the immunofluorescent antibody technique (8, 16). Granulocyte-specific ANF have been demonstrated mainly in sera from patients with arthritic lesions, especially rheumatoid arthritis (10), though absorption studies also have revealed the existence of granulocyte-specific ANF in sera from patients with systemic lupus erythematosus, containing apparently organ-non-specific ANF reacting with all kinds of nuclei (6).

Wide variations in the incidence of ANF reacting with leucocytes in human blood smears have, however, been reported (Feltkamp (11) 12 per cent, Barnett *et al* (2) 30 per cent; Hijmans *et al* (13) 41 per cent, Alexander *et al* (1) 65 per cent, Hasker *et al* (12) 70 per cent, Elling (7) 75 per cent), but since special precautions have to be taken to ascertain the maximum antigenicity of granulocytes, this may probably explain the marked differences found. The main point seems to be that alteration of the granulocytes, *e.g.* by freezing the smears prior to testing for ANF, caused a considerable increase of the incidence of granulocyte-reactive ANF in sera from patients with rheumatoid arthritis. Thus in a previous report (7) it was found that while 75 per cent of the sera from patients with rheumatoid arthritis reacted with altered granulocytes, only 30 per cent of the same sera reacted with unaltered leucocytes. Similarly, only 35 per cent of the sera reacted with other human nuclei indicating that ANF in rheumatoid arthritis exhibit a limited nuclear reactivity, mainly directed against the polymorphonuclear granulocytes.

Since the increased reactivity of altered granulocytes might indicate an inaccessibility of the living intact leucocytes to some ANF, the present study was conducted with the object of investigating the inter

action of ANI with polymorphonuclear granulocytes of various ages and degrees of alteration employing sera containing, at present, only granulocyte-specific ANI is the analytical "tool." Since inaccessibility of the intact leucocytes may be related to the molecular size of the antibody (11) special emphasis has been laid on the reaction of leucocytes with ANI of different molecular weights.

MATERIAL AND METHODS

The material consisted of 20 sera selected to contain ANI reacting with polymorphonuclear granulocytes only, as determined by the immunofluorescent antibody technique. To ascertain the organ specificity the 20 sera were tested with the following human tissues: thyroid gland, normal liver, gastric mucosa, striated muscle and renal cortex.

Fifteen sera were derived from patients with rheumatoid arthritis, one serum from a patient with Felty's syndrome, 2 sera from patients with systemic lupus erythematosus and 3 sera from patients with other non-rheumatic diseases.

Methods. The fluorescent antibody technique for the determination of ANI was performed as previously described (6, 9, 10) with the following modifications.

Blood smears used as nuclear sources were prepared from the same human donor in 3 different ways: 1) Blood smears were prepared immediately after drawing the blood and the sera were applied to the smears within 15 minutes; 2) blood smears were prepared as in 1) but immediately frozen by placing them on dry ice for a few minutes and then thawed at room temperature repeated once or twice; 3) after drawing the blood was shaken vigorously for 10 minutes in tubes containing glass beads. Smears were then prepared as in 1).

Rabbit antisera against human gammaglobulins were prepared and conjugated with fluorescein isothiocyanate as described (9). Specific antisera against human Ig G, Ig A and Ig M immunoglobulins were obtained from the Central Laboratory, Roellebruik, Amsterdam and conjugated with fluorescein isothiocyanate. The immunological specificity of these antisera was tested as described previously (6).

The sulphhydryl sensitivity of ANI was determined as described (10) with the use of 1-methyl-2-cysteine. Controls were diluted in buffered saline.

EXPERIMENTS AND RESULTS

Nuclear Reactivity of Blood Smears of Various Ages

With the use of blood smears less than 15 minutes old 18 of the 20 sera employed reacted each time tested (Table 1); while 2 sera were positive in 1 out of 3 tests only. Repeating the reactions 5-6 times with each of the 18 positive sera they were found to react in every test showing that the use of fresh blood smears yielded fairly reproducible results.

With the use of blood smears 30 minutes old altogether 6 sera became negative including the 2 sera showing varying positive and negative reactions with 15 minute-old smears. Employing blood smears 60 minutes old the 6 sera were still positive but 2 other sera showed inconstant positive and negative results.

The finding that the same 6 sera reacted constantly negative when 30 and 60 minute-old smears were used as nuclear sources indicates that the non reactivity is not entirely dependent on changes of the leucocytes owing to age but might be related to the serum factors concerned.

higher molecular weight, for example. The finding that the antigenicity of these old smears was restored for 4 of the 6 negative sera by freezing the 60-minute-old smears prior to testing for ANF, supports this view.

TABLE 1

Nuclear Reactivity of Human Blood Smears of Various Ages with 20 Sera Containing Granulocyte Specific ANF

	Human blood smears								
	15 minutes old			30 minutes old			60 minutes old		
	pos	neg	dubious pos.	pos	neg.	dubious pos.	pos	neg	dubious pos.
Number of sera	18/20	0/20	2/20	14/20	6/20	0/20	12/20	6/20	2/20
Number of tests performed with each serum	5 6	-	3	1	1	1	2	2	2

TABLE 2

Effect of Alteration of Blood Smears Prior to Testing for Granulocyte-Specific ANF in 20 Sera Compared with the Results Obtained with Fresh Unaltered Blood Smears

Human blood smears					
Unaltered 15 minutes old		Altered by freezing $\times 3$		Altered mechanically	
pos	neg	pos	neg	pos	neg
20/20	2/20*	19/20	1/20	8/8	0/8

* 2 sera inconstantly positive

Nuclear Reactivity of Leucocytes Altered Mechanically or by Repeated Freezing and Thawing

The reaction of sera with leucocytes altered by freezing, compared with the reaction of sera with 15-minute-old, unaltered leucocytes, is seen in Table 2. Of the 20 sera containing leucocyte-specific ANF 17 reacted equally well with blood smears frozen and thawed 3 times as with fresh, intact leucocytes. The 2 sera reacting inconstantly with unaltered leucocytes showed positive reactions with altered leucocytes while one serum did not react with altered leucocytes. To obtain maximum antigenicity or accessibility, freezing and thawing the blood smears only one or two times proved insufficient, since only 12, respectively 14 out of 19 sera tested gave positive reactions. All of 8 sera tested against leucocytes altered by shaking the blood with glass beads reacted, thus showing that a simple mechanical alteration of the leucocytes produced the same optimal antigenicity as repeated freezing did.

Determination of the Immunoglobulin Classes of Granulocyte-Specific ANF, Employing Leucocytes Altered in Various Ways

To test whether the non reactivity of old leucocytes to some ANF found in the present study was in any way related to the molecular size of the antibody the immunoglobulin classes of granulocyte-specific ANF were determined by the use of blood smears altered in various ways and employing specific, conjugated antisera against human Ig G, Ig A and Ig M immunoglobulins.

TABLE 3

Immunoglobulin classes of Granulocyte-Specific ANF in 20 Sera Tested Against Altered and Unaltered Leucocytes as Nuclear Antigen

Antigen	Immunoglobulin class		
	Ig G	Ig A	Ig M
15 min old unaltered blood smears	17/20 (11)	4/20 (1)	5/20 (2)
Blood smears altered by freezing $\times 3$	17/20* (2)	7/20* (0)	15/20 (2)

In brackets: Number of sera containing ANF of one immunoglobulin class only
* 1 serum constantly negative

Table 3 shows that with the use of 15 minute-old unaltered leucocytes 17 of the 20 sera contained Ig G ANF, 4 contained Ig A ANF and 5 sera contained Ig M ANF. 14 of the sera thus contained ANF belonging to one immunoglobulin class and 6 sera contained ANF of more than one immunoglobulin class.

Employing blood smears altered by freezing 3 times 3 more sera were found to contain Ig A ANF and 10 more sera to contain Ig M ANF, while the frequency of low molecular weight Ig G ANF was exactly the same as found with the use of 15 minute-old unaltered leucocytes. Only 1 sera now contained ANF of one immunoglobulin class.

The results thus show that the use of altered leucocytes increased the detection of high molecular weight ANF while ANF of low molecular weight (Ig G) reacted with intact as well as with altered granulocytes to the same extent.

The question arises that the high molecular weight ANF found to react with altered granulocytes only might not be organ specific and might react with other nuclei altered in the same way. This was partially excluded since none of 8 sera tested reacted with thyroid nuclei altered by repeated freezing and thawing and no reactions were observed with lymphocytes treated in the same way as the granulocytes.

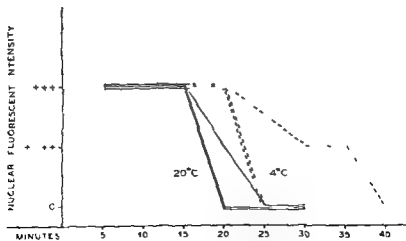
Sulphydryl Sensitivity of Leucocyte Specific ANF in 6 Sera and of Organ Nc

Patients	C			N			N J		
	Ig			Ig			Ig		
Immunoglobulin classes	G	A	M	G	A	M	G	A	M
Nuclear activity before	+++	0	0	+++	0	0	0	0	+++
and after treatment with dimethylcystein	+++	0	0	+++	0	0	0	0	0

Sulphydryl Sensitivity of Granulocyte-Specific ANF

To test the correlation between the sulphydryl sensitivity of granulocyte-specific ANF and the immunoglobulin classes of ANF as determined by the use of specific antisera against human Ig-G, Ig A and Ig-M immunoglobulin classes, 6 sera were selected from the 20 containing granulocyte-specific ANF. Two sera contained apparently only Ig-G ANF, 2 only Ig-M ANF, and 2 contained ANF of both Ig G and Ig-M specificity. Furthermore, as a control, one serum from a patient with S L E, containing organ-non-specific ANF belonging to the three immunoglobulin classes and with a titre of 1:400 (tested with use of a conjugated antiserum against all human gamma-globulins and with use of thyroid nuclei), was tested for ANF before and after treatment of sera with dimethyl-cystein.

Table 4 shows a fair accordance between the sulphydryl sensitivity and the immunoglobulin classes as determined by the use of specific conjugated antisera. The Ig-G ANF was unaffected except for a small

*Fig 1*

Nuclear reactivity of blood smears of various ages with 3 sera containing granulocyte specific ANF. The reactions were performed at 20° C and 4° C.

Specific ANF in I Serum (H) as Determined with Conjugate I Specific Antiserum

M			S ₁			J			B		
Ig M			Ig M			Ig M			Ig M		
0	0	+++	+++	0	+++	+++	0	+++	+++	+++	+++
0	0	0	++	0	0	++	0	0	+++	+	+

decline in the intensity of the fluorescence in 2 sera. The Ig M ANF activity was abolished entirely except in the case of the lupus serum in which however a considerable fall in the intensity of the fluorescence was observed. The Ig A ANF showed a similarly great decrease in fluorescent intensity after incubation with dimethyl cysteine.

Effect of Varying the Reaction Temperature

Eight sera containing granulocyte specific ANF were incubated with unaltered blood smears 15 minutes old at 1°, 20° and 37° C, for half an hour. No difference in nuclear staining was found but Fig. 1 shows that performing the reaction at a low temperature increased the limiting time factor from 15 minutes to 35 minutes in one case.

DISCUSSION

The interaction of granulocyte specific ANF with polymorphonuclear granulocytes in blood smears has been shown in the present study to depend partly on the age of the slides but chiefly on the degree of damage which the leucocytes underwent prior to testing for ANF. The three kinds of blood smears used as nuclear antigen: unaltered blood smears less than 15 minutes old, blood smears altered by repeated freezing and thawing and leucocytes altered mechanically by shaking the blood with glass beads were shown to be nearly equally fitted for the determination of granulocyte specific ANF while unaltered blood smears more than 15 minutes old and leucocytes altered to a lesser degree were found less reactive. Thus both were found which invariably showed no nuclear staining with 60 minute old blood smears indicating that the use of fresh blood smears is important to secure the optimal antigenicity or accessibility of leucocytes.

Although the use of fresh non-altered leucocytes yielded constant and reproducible results further investigations showed that the degree of alteration which the leucocytes underwent before testing for ANF played an important role in determining the immunoglobulin class

of ANI reacting with granulocytes therefore this is also of significance for the estimation of the frequency of ANI in human sera. While sera with low molecular weight ($I_g G$) ANI reacted with the fresh intact granulocytes as well as with altered granulocytes many more sera proved to contain ANI of high molecular weight when altered leucocytes were used as nuclear antigen. Thus only one third of sera with $I_g M$ ANI and only half of sera with $I_g A$ ANI which were found to react with altered granulocytes combined with an altered 15 minute old granulocytes suggesting that the intact (living) granulocytes are inaccessible to most ANI of high molecular weight.

The possibility that the non reactivity of old blood smears to certain ANF is caused not by any degradation of the nuclear antigen but by an increasing inaccessibility of the leucocytes with age is suggested by the fact that the reactivity of old blood smears could be restored to some extent by freezing and thawing the smears and by the finding that it was always the same 6 out of 20 sera which did not react with leucocytes in 60 minute old blood smears. There is however no direct evidence that the lack of reactivity of old smears is related to the molecular size of the antibody since some sera even of $I_g G$ specificity did not react with 60 minute old unaltered blood smears. It is however conceivable that the ANI in these 6 sera which did not react with old blood smears formed some kind of high molecular weight complexes e.g. with rheumatoid factors (15).

Inaccessibility of cells to antibodies has been demonstrated before in a few cases. The nuclei of sperm have been shown not to react with ANF as the nuclear membrane is not accessible to molecules with a molecular weight more than 60 000 (14) which is considerably under the molecular weight of even $7 S$ antibodies. Since mechanical alteration of leucocytes prior to testing for leucocyte factors in human sera causes a considerable increase in the sensitivity of the test (17) this might indicate a similar inaccessibility of unaltered leucocytes to leucocyte factors. The procedure of freezing and thawing used in the present studies to disrupt the cells is identical with the method employed when tissues are made accessible to high molecular weight complexes of antibodies conjugated with ferritin (5). When working with ferritin labelled antibodies it has been necessary to freeze and thaw the tissue 3 times to obtain maximum accessibility. This is in keeping with the result obtained in the present study.

The finding that high molecular weight granulocyte specific ANF chiefly reacted with altered granulocytes may probably explain the wide variation in the frequency of *granulocyte reactive* ANI found previously in rheumatoid arthritis (Review see 7). The use of altered granulocytes as nuclear antigen has thus been found to increase the incidence of ANI to 75 per cent as compared with only 30 per cent reacting with intact fresh leucocytes (7). Since ANI in rheumatoid arthritis are mostly high molecular weight antibodies (2, 1) this in

dictates that the increased frequency of ANF found in rheumatoid arthritis with the use of altered granulocytes is caused by ANF of high molecular weight.

SUMMARY

The interaction of 20 sera containing granulocyte specific ANF with polymorphonuclear granulocytes of various ages and degrees of alteration has been investigated.

The use of 15 minute-old unaltered granulocytes was shown to give reproducible results while the use of older leucocytes invariably rendered some sera negative probably owing to an increasing inaccessibility of leucocytes with age since alteration of the cell by repeated freezing restored the reactivity of old blood smears to some extent.

In further investigations of the inaccessibility of granulocytes to some ANF it was shown that while all sera with ANF of low molecular weight (Ig G) reacted with unaltered fresh granulocytes only one third of sera with Ig M ANF and only half of sera with Ig A ANF combined with unaltered granulocytes this suggests that the living intact granulocytes are inaccessible to most granulocyte specific ANF of high molecular weight. To obtain maximum accessibility it was found necessary to alter the cells by repeated freezing and thawing.

This finding may probably explain the wide variation in the frequency of *granulocyte reactive ANF* previously found in sera from patients with rheumatoid arthritis since alteration of granulocytes increases the incidence of ANF in rheumatoid arthritis considerably and since ANF in rheumatoid arthritis are mostly high molecular weight antibodies.

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HISTOCHEMICAL APPLICATION OF RUTHENIUM RED IN THE STUDY OF MAST CELL ULTRASTRUCTURE

By

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It is now generally agreed that the metachromatic substance in mast cell granules is heparin. This concept is based upon a relationship between mast cell and heparin content of tissues (Jaffes *et al.* 1937) as well as determination of heparin content of isolated mast cells (Schiller & Dorfman 1959, Bloom & Ringertz 1960) and isolated mast cell granules (Parekh & Gluck 1962, Logunoff *et al.* 1964). While the behavior of mast cell histamine during many pathological conditions is well documented, the fate of granule heparin is more obscure (Riley 1959, Bloom 1965). Recently it has been shown that rat mast cell granules contain 316 μ g/mg dry weight heparin and that the removal of heparin from isolated granules by salt extractions gives no recognizable granule structure left (Logunoff *et al.* 1964). As heparin obviously constitutes such an integral part of the granule, there is some reason for methods making it possible to follow changes in mast cell heparin in anaphylactic as well as cytotoxic reactions.

Recently Luft (1964) reported that the inorganic dye Ruthenium red $\text{Ru}(\text{OH})\text{Cl}_2\text{N}_2\text{H}_2\text{SO}_4 \cdot 3\text{H}_2\text{O}$ easily used to stain pectin substances binds to and precipitates several animal acid mucopolysaccharides. Luft also found that Ruthenium red is sufficiently electron dense to be localized ultrastructurally.

These properties of Ruthenium red led us to investigate the possibility of developing a method using this stain for visualizing the mast cell heparin in light and electron microscopy. Considering the possibility that the acid mucopolysaccharide staining properties of Ruthenium red may be related to its many ammonium groups, it seemed reasonable to expect that precipitation of the saccharide polyanions with long chain aliphatic ammonium salt (Scott 1960) would interfere



Fig 1

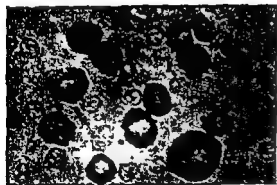
Tissue mast cell from hamster cheek pouch vital stained in 0.05 per cent Ruthenium red fixed in glutaraldehyde and sectioned in a cryostat microtome $\times 800$

Fig 2

Rat peritoneal mast cells treated with cetylpyridinium chloride before staining with Ruthenium red and fixation in glutaraldehyde. Evidently, staining of the granules of mast cells is blocked by the quaternary ammonium salt. The corresponding result in the electron microscope is illustrated in Fig 12 $\times 800$

Fig 3

Rat peritoneal mast cells treated as described in Fig 2 but in addition stained red with ferric thiocyanate $\times 800$



Figs 4-5

Fig 4 Tissue mast cell from hamster cheek pouch. Fixed in glutaraldehyde containing 0.05 per cent Ruthenium red. Fixed fixation in osmium tetroxide. All mast cell granules are of almost equal electron density contrasting to the pale nucleus. Uncontrasted as all subsequent figures $\times 9700$

Fig 5 Detail from the same mast cell as in Fig 4 the granules of which are very electron dense with a fine fibrillar mesh $\times 4000$



with a successive staining with Ruthenium red. Consequently an attempt was also made to assess the selectivity of the dye for the granule heparin by blocking with cethylpyridinium chloride.

MATERIALS AND METHODS

Mast cells were isolated from the peritoneal fluid of 20 male and female Sprague-Dawley rats weighing 200-300 g. In pilot studies the following procedure to obtain intact mast cells was experienced as the most convenient. Five ml of a 0.1 M phosphate buffer, pH 7.4 containing 7.5 per cent sucrose was injected into the peritoneal cavity of animals anaesthetized with ether. Heparin was avoided as it might interfere with the staining. In some experiments we used a Krebs Ringer solution without calcium (143 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 3.1 mM phosphate buffer, final pH 7.2), containing in addition sodium citrate at a concentration of 15.5 mM. After gentle massage for 15 min the abdomen was opened and the fluid withdrawn. An equal amount of a 4°C 0.1 per cent solution of Ruthenium red in the aforementioned phosphate buffer was immediately added. The specimen tube was placed in an ice bath for 5 min and centrifuged at $30 \times g$ for 10 min at 4°C. The cell sediment was fixed for one hour in 3 per cent phosphate buffered glutaraldehyde at 4°C, centrifuged at $300 \times g$, rinsed in 0.1 M phosphate buffer, dehydrated and embedded in Epon 812 according to Luft (1961). In some cases the cell suspensions were in addition postfixed in Villonig's phosphate buffered OsO_4 for one hour (Villonig 1962). The temperature was not allowed to rise above 4°C until infiltration with the epoxy resin.

In addition tissue mast cells were studied in hamsters, the cheek pouches of which are rich in mast cells and easy of access (Gustafson & Cronberg 1963). The animals weighing 80-110 g were anaesthetized with ether and the cheek pouches exposed to 0.05 per cent Ruthenium red in a 3 per cent phosphate buffered glutaraldehyde solution. At the same time 2 ml of the same solution was injected intracardially until the animal was killed. The cheek pouches were excised, cut in thin slices and placed in the same stain fixative solution for one hour at 4°C and washed for another hour in the phosphate buffer. Further treatment for electron microscopy was carried out as described for rat peritoneal cells. In addition 10 μ thick sections were cut with a cryostat microtome and without further treatment mounted in polysorbital.

From the Epon blocks 1 μ thick sections for light microscopy as well as ultrathin ones were cut with an LKB ultramicrotome. Generally contrasting was avoided. The ultrathin sections were examined in a Siemens Elmiskop 1A at 60 kV.

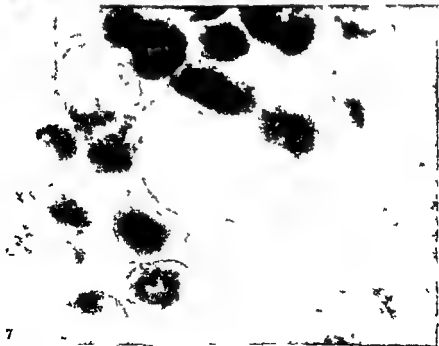
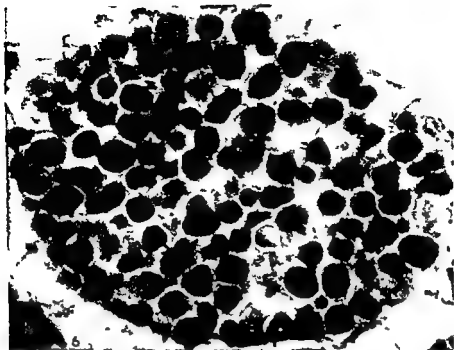
Control and Selectivity Testing Procedures

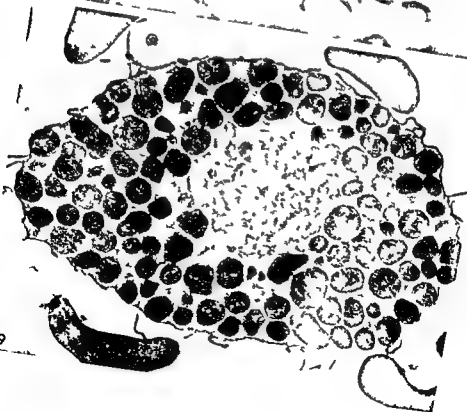
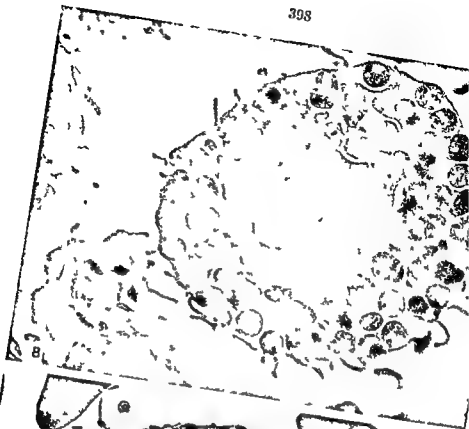
A paired series of sections corresponding to those above were processed in the same fashion but without using Ruthenium red. In order to block the Ruthenium red staining some specimens were exposed to a 0.05 per cent solution of cethylpyridinium chloride (1 hexadecylpyridinium chloride Merck AG Darmstadt Germany) in 0.05 M tris maleate buffer containing 8.72 per cent sucrose and in one series also 3.9 per cent NaCl in order to increase ionic concentration (Zugibe & Fink 1967b).

Figs 6-7

Fig 6 Rat peritoneal mast cell vital stained in 0.05 per cent Ruthenium red and fixed in glutaraldehyde. There is a considerable difference in electron density between the granules. The distribution of the various types is rather even all over the cytoplasm. $\times 11,000$.

Fig 7 Detail of a rat peritoneal mast cell treated as for Fig 6. The electron dense granules are rather homogeneous and often smaller than the lighter ones which show a fine fibrillar mesh. To the right part of the picture the cell nucleus is seen. $\times 43,000$.





Some of the cell suspensions were then exposed to Ruthenium red and treated as described above for light and electron microscopy. Other cell suspensions exposed to ethylviridium chloride were dehydrated, embedded, cut in 1 μ thick sections and exposed to a solution of 0.1 per cent ferric thiocyanate $\text{Fe}(\text{SCN})_3$ (Zugbe & Fink 1966a). In 0.1 M tris-maleate buffer containing 2% per cent sucrose. The use of tris-maleate buffer was found necessary as ferric thiocyanate in the concentration used was found insoluble in phosphate buffers with a corresponding ionic strength.

RESULTS

After vital staining, the tissue mast cells appeared intensely red in the cryostat microtome sections. In contrast other structures were unaffected or had at most, got only a faint tinge (Fig. 1). It was evident that the selective staining of mast cell granules with Ruthenium red corresponded with the metachromasy produced by toluidine blue solutions. Corresponding results were obtained with 1 μ thick frozen sections of the peritoneal mast cells the granules of which during the whole process had retained a strong Ruthenium red stain.

In the electron microscope equivalent results were obtained (Figs. 4 and 5). While nuclei as well as other cell organelles and cytoplasm were of low electron density in the uncontrasted sections, the mast cell granules in comparison were markedly electron dense indicating a selective binding to the latter. When post fixation in osmium tetroxide was avoided the considerable variation in electron density between different granules is striking (Figs. 6 and 7). The least electron dense granules were often large and similar to each other in structure, presenting a fine granular meshwork. The more electron dense granules were more varying in size and showed patches of electron dense material against a homogeneous background.

In contrast granules only treated with glutaraldehyde varied little in electron density (Fig. 8) as was also the case if post fixation in osmium tetroxide was carried out in addition (Figs. 9 and 10). With the latter technique membranes were distinct while Ruthenium red alone merely stained the contents of the granules leaving membranes hardly discernible.

In cells treated with the Ruthenium red method and post fixed in osmium tetroxide membrane structures were distinct and the dark patches resulting from the Ruthenium red recognizable (Fig. 11). Thus

(Figs. 8-11)

Fig. 8. Unstained rat peritoneal mast cells in glutaraldehyde. There is little difference in electron density between different granules and between these and the cell nucleus and cytoplasm. $\times 11,200$.

Fig. 9. Rat peritoneal mast cell treated as in Fig. 8 and in addition post fixed in osmium tetroxide. The mast cell granules are of about equal electron density. Only the contrast between granules and cytoplasm as well as cell nucleus enhanced. The contrast is practically normal under the granules. A few mitochondria can be seen. $\times 10,000$.

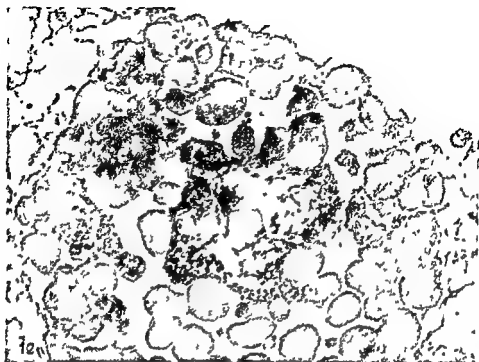


Fig. 12

This peritoneal mast cell was treated with ethylpyridinium chloride before staining with Ruthenium red. Fixation in glutaraldehyde, no post fixation. Cell nucleus and cytoplasm are severely affected by the quaternary ammonium compounds (no NaCl was added). Only a few granules to the left of the cell nucleus show some electron dense traces ascribable to Ruthenium red while the other granules are very pale although they do not seem to have increased very much in size. $\times 14,400$.

This figure should be compared with Figs. 6 and 7.

biological state of different granules. If this is the case, granules of normal mast cells may represent a rather heterogeneous population from a functional point of view.

Obviously, there are disadvantages of using a vital Ruthenium red staining in electron microscopy. These are incomplete reproduction of membranes and nuclei and a tendency of mast cells to attract small contaminating particles. We think, however, that the staining of granule heparin makes it possible to follow granule changes during pathological conditions. If this is the main interest, the mast cells ought to be vital stained with Ruthenium red. For such purposes post fixation in osmium tetroxide and/or contrasting should be avoided.

SUMMARY

A method has been developed using the inorganic dye Ruthenium red in the study of mast cells for light and electron microscopy. Ruthenium red was found to react selectively with mast cell granules resulting in

a stable red complex. In the electron microscope equivalent results were obtained granules being intensely electron dense. After vital staining with Ruthenium red followed by glutaraldehyde fixation mast cell granules were found to present great variations in electron density. By blocking the granule heparin with ethylpyridinium chloride the staining with Ruthenium red was inhibited. It is concluded that Ruthenium red stains mast cell granule heparin.

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COMPLEMENT-FIXING LIVER ANTIBODIES

2 Occurrence in a Material of Patients with Chronic Hepatitis

By

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In recent years the serology of acute and chronic hepatitis has been the object of several investigations. Before 1957 these were mainly undertaken in the search for a specific serologic test for the diagnosis of acute viral hepatitis. Since then, the investigations have chiefly been concerned with the serology of chronic hepatitis as the detection of liver-autoantibodies in the sera of such patients seemed to provide an explanation of the pathogenesis of the disease.

Based on experiences with the complement fixation test earlier investigators have suggested that a liver damage arisen during the acute phase of infectious hepatitis might evoke a production of antibodies directed against the patient's own liver, thus bringing about the development of chronic hepatitis (Eaton *et al* 1944, Bjørneboe & Aagaard 1947, Gear 1955).

During the course of investigations aimed at detecting infectious hepatitis virus in livers from patients who had died from acute yellow atrophy of the liver, Gajdusek (1958) observed a complement fixation with suspensions of liver tissue as antigen in sera from 6 out of 75 (8 per cent) patients with acute viral hepatitis and from 22 out of 34 (67 per cent) patients with various forms of chronic hepatitis. In the latter group of patients complement fixing liver antibodies were demonstrated in 7 out of 9 cases with chronic active hepatitis, in 3 out of 5 cases with lupoid hepatitis—a severe form of chronic active hepatitis associated with positive L.C. cell tests (MacLag *et al* 1956)—in 7 out of 11 cases of chronic nutritional hepatitis, and in 3 out of 8 cases of biliary cirrhosis. High titres of liver antibodies were found in three of the patients with lupoid hepatitis and in one patient with primary biliary cirrhosis. The complement fixing antibodies were thermostable (60° C. for 30 min.) migrated at paper electrophoresis with the slow moving gamma-globulin fraction, and were precipitated from serum with ammonium sulphate. Gajdusek therefore regarded them as true antibodies but did not attribute any pathogenic significance to their presence in serum since they were neither organ- nor species-specific.

nor could they be demonstrated in sera from all patients with chronic hepatitis (Gajdusek 1958).

This concept of the pathogenic importance of the complement fixing liver antibodies seemed to be further supported by observations reported by Mackay *et al* (1957) and by Mackay & Larkin (1958). These investigators found that serum of one case with lupoid hepatitis, one with nutritional cirrhosis, two with lupus erythematoses disseminatus and one with microglobulinemia failed to react with antigen prepared from the patient's own (autologous) organs and tissues (liver, kidney, spleen and striated muscular tissue), although the same sera gave high titres with antigens prepared from the above mentioned organs and tissues obtained at necropsy from other patients. In a later study (Mackay & Larkin 1959) another 10 patients (3 with active chronic hepatitis, 2 with lupoid hepatitis, 1 with nutritional hepatitis, 3 with lupus erythematoses disseminatus and 1 with colitis ulcerosa) whose sera in all cases fixed complement with heterologous organs and tissues were examined for complement fixing antibodies to antigens prepared from autologous organs and tissues (liver, kidney and muscle). Sera from 3 of these 10 patients failed to react with autologous organs and tissues while in the remaining 7 cases a positive complement fixation was observed.

The problem of the pathogenic significance of the complement fixing tissue antibodies remains undetermined. Their frequent occurrence in primary biliary cirrhosis and in chronic passive hepatitis seems, however, to suggest that immunologic processes may play a role in the pathogenesis of these types of cirrhosis (Mackay 1958, 1960; Decker *et al* 1960; Gokcen 1962; Pasnick *et al* 1962; Walker *et al* 1963). In other forms of cirrhosis where the aetiological agent must be considered known (alcohol, obstruction of the biliary tract owing to calculi, cancer etc.) complement fixing liver antibodies occurred with the same frequency only as in normal individuals (Mackay & Larkin 1958; Gokcen 1962; Walker *et al* 1963). Consequently the test for tissue antibodies has been considered of some value in the differentiation between primary biliary cirrhosis and extrahepatic obstructive jaundice (Gokcen 1962; Walker *et al* 1963).

The aim of the present study has been to determine the frequency of occurrence of complement fixing tissue antibodies in sera from patients with chronic hepatitis and to assess the diagnostic value of this test.

MATERIAL AND METHODS

The material comprised 135 patients with liver pathology. In all cases the diagnosis was made on the basis of clinical, laboratory and histological findings. The patients were divided into two groups: 1. Patients with chronic hepatitis (n = 76). 2. Patients with other liver diseases (n = 59).

In the first group of under 50 the number of men and women was equal 76 (50 per cent of the patients were over 60 years of age). Based on anamnestic data the patients

Table 1
Results of Complement Fixation Tests with Liver, Thymus and Kidney Antigens
in Sera of Patients with Chronic Hepatitis and Normal Blood Donors

Organs Used for the Antigen Preparations	135 Patients with Chronic Hepatitis				Normal Blood Donors			
	No of Sera Tested		67 Sera Tested with all Antigen Types		122 Sera Tested with all Antigen Types		Percentage Positive	
	No of Sera Tested	No of Sera Positive Titre 8-16 \geq 32	Percentage Positive	No of Sera Positive Titre 8-16 \geq 32	No of Sera Positive Titre 8-16 \geq 32	No of Sera Positive Titre 8-16 \geq 32	Percentage Positive	Percentage Positive
Normal Human Livers (Biopsy)	120	16	12.5	19	5	5	—	4.1
Normal Livers from Rheumatic Nephrosis	108	10	25.0	18	5	5	—	4.1
Normal Human Livers (Necropsy)	92	7	15.2	11	5	5	—	4.1
Thyroidic Human Thymus Glands (Biopsy)	135	11	14.1	10	5	5	—	4.1
Normal Human Kidney (Necropsy)	128	17	18.8	17	6	6	—	4.9

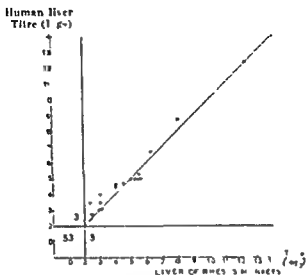


Fig. 3

Relationship between the Titer Values Obtained with Suspensions of Normal Human Biopsy Liver and Normal Liver of Rhesus Monkeys in Sera of 93 Patients with Cirrhosis of the Liver

glands) were also positive with liver and/or kidney antigen thus organ specific complement fixing thyroid antibodies were demonstrated in 4 cases of chronic hepatitis (only one of these had disease of the thyroid gland). A total of 67 sera from patients with hepatic cirrhosis and 122 sera from normal donors were tested with all the antigen types shown in Table 1. As it appears from this reduced material of patients the frequency of positive tests with antigens from normal biopsy liver, normal liver of rhesus monkeys and normal human kidney tissue obtained at necropsy was almost the same (20.4-28.4 per cent) and higher than that given by antigens from normal human necropsy liver and thyrotoxic thyroid glands (14.9-16.4 per cent), whereas in the donor material a positive reaction was equally frequent (41-49 per cent) with all the types of antigen.

A total of 93 sera from the patient material was tested with antigens from normal human biopsy liver as well as from normal liver of rhesus monkeys. The results are shown in Table 1. A positive complement fixation with the two types of antigen was demonstrated in 26 sera. 66 sera were negative with both antigen types and only one serum was positive with biopsy liver and negative with liver from rhesus monkeys. Normal human biopsy liver and normal liver of rhesus monkey thus seem to react nearly identically in the complement fixation test.

Fig. 4 shows the incidence of positive reactions with antigens from normal human biopsy liver and/or normal liver from rhesus monkeys in the previously mentioned 4 types of cirrhosis. It will be seen from the

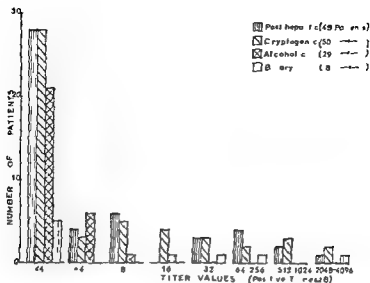


Fig. 4

Distribution of Titer Values of Complement Fixing Antibodies to Normal Human Biopsy-Liver and Normal Liver of Rhesus Monkeys in the Various Types of Chronic Hepatitis

figure that positive reactions occurred with the greatest frequency (33–38 per cent) and with the highest titres in the groups of patients with posthepatic and cryptogenic cirrhosis, and that these two types of cirrhosis differed neither in respect of the frequency of positive tests nor as to the height of the liver antibody titres. Of 8 sera from patients with biliary cirrhosis three were positive. Of these two sera giving high liver antibody titres were from patients with primary biliary cirrhosis. In the group of alcoholic cirrhosis it is noteworthy that only two out of 29 (6.2 per cent) sera fixed complement in the presence of liver tissue in both cases with low titres (8 and 16) and that a relatively large number of sera (6 out of 29) had titres of 4.

Table 2 gives the results of complement fixation tests with liver, thyroid and kidney antigen on sera from 27 patients with chronic hepatitis. It will be seen from the table that all sera except two (Nos. 14 and 15), having a high titre (≥ 64) of liver antibody also reacted positively with antigens from thyroid and/or kidney tissue—a result which seems to suggest a certain relationship between high contents of antibody in serum and degree of organ reactivity. With the exception of 4 sera (Nos. 1–4) which gave high titres with all the antigens listed in the table, there was no relationship between the height of the liver antibody titres and those of the thyroid and kidney antibody titres, and no relationship between the results obtained with thyroid and kidney antigens. Of 13 sera (Nos. 1–13 in Table 2) which fixed complement in the presence of liver, thyroid and/or kidney tissue 10 were from females and 3 from males.

Results of Cysticercus Celli in Test with Antigens for Cell or Throat and Liver Tissue in Sera for Cell or Throat and Liver Tissue

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Serial No.	Age (yr)	Sex	Normal Liver Tissue		Throat Tissue		Throat Tissue		Normal Kidney Tissue
			Human	Neer 1st	Rhesus Monkey	Human	Human	Human	Neer 1st
1	64	♀	4096	512	4096	2048	2048	512	512
2	69	♀	2048	512	2048	2048	2048	2048	2048
3	65	♀	256	72	256	256	256	128	128
4	57	♀	71	8	71	71	71	8	8
5	70	♂	72	8	16	16	16	8	8
6	49	♀							
7		♀	128	8	128	128	8	8	8
8	7	♀	128	16	71	71	71	71	71
9	7	♀	72	8	72	72	72	72	72
10	64	♂	32	32	72	72	16	8	8
11	2	♀	32	32	32	32	32	32	32
12	67	♂	32	32	32	32	32	32	32
13	61	♀	16	16	16	16	16	16	16
14	54	♀	1024	128	1024	1024	1024	1024	1024
15	37	♀	1024	8	1024	1024	1024	1024	1024
16	75	♀	32	32	32	32	32	32	32
17	69	♀	16	16	16	16	16	16	16
18	78	♀	8	8	8	8	8	8	8
19	57	♂	8						
20	80	♀					8	8	8
21	69	♀							
22	51	♀							
23	49	♀							
24	71	♀							
25	60	♀							
26	76	♂							
27	58	♂							

TABLE I
 Sex and Age Distribution of Positive Reactions with Normal Human Biopsy Titer and Normal Titer from Rhesus Monkeys
 as Antigen in Sera from 135 Patients with Chronic Hepatitis and from 238 Normal Blood Bank Donors

Age (yr)	Sex	135 Patients with Chronic Hepatitis				238 Normal Blood Bank Donors			
		No of Cases Tested	No of Cases Positive	Expected No of Cases Positive Corresponding to the % of Normal Reactors	Positive %	No of Cases Tested	No of Cases Positive	Positive %	
18-40	♂	2				99	2	23	
	♀	2	1			32	1		
41-50	♂	9	1	03	33.3	32	2	37	
	♀	9	5	03		22			
51-70	♂	25	7	10	30.9	18			
	♀	56	18	21		35	2	38	
71-90	♂	6			25.0				
	♀	26	8						
	♂	42	8	12	29.6	149	4	29	
	♀	63	32	27		89	3		

Table 3 shows the sex and age distribution of positive reactions with antigens from normal biopsy liver and liver of rhesus monkeys in the patient material and in the donor material. The frequency of positive tests was greater in the patient material than in the donor material, a result which is further stressed by a comparison of the number of positive cases found with the number of positive cases expected corresponding to the percentage positive of the donor material. In the patient material a positive test occurred more frequently in females than in males in the age groups 11-30 and 71-90, and with almost the same frequency in either sex in the age group 51-70 years. A rise or fall in the frequency of positive reactions with increasing age in either sex was not found.

Sera from three of the patients with chronic hepatitis were examined for complement fixing antibodies against their own (autologous) liver tissue which in all three cases was obtained surgically. The results are shown in Table 4 which further shows the titres obtained with isologous cirrhotic and isologous normal liver tissue as antigen. In two of the cases (I Jo and I J) complement fixing antibodies to autologous liver tissue were demonstrated while one case (K G) failed to fix complement in the presence of autologous liver tissue. Complement fixing antibodies to suspensions of isologous cirrhotic and isologous normal liver tissue were demonstrated in sera of all cases except one (case I J)—the serum of which failed to fix complement with the liver antigen from case K G. The small amount of liver tissue available precluded further examination of these varying results.

TABLE 4

Results of Complement Fixation Tests in Sera from three Patients with Chronic Hepatitis Using Isologous Autologous and Isologous as well as Normal Isologous Liver Tissue as Antigen

Type of Cirrhosis	Serum Case	Antigens (1:10) (1:100) (1:1000) (1:10000) (1:100000)	Antibodies (Normal) (Isologous) (Isologous)
Posthepatic	K G	+	51%
Cryptogenic	I J	+	12
Primary Biliary	F J	+	12

I

Several investigators have demonstrated complement fixing antibodies to liver tissue in sera from patients with various forms of chronic hepatitis, most notably in posthepatic (postviral) cirrhosis, alcoholic cirrhosis, and cryptogenic cirrhosis. In chronic hepatitis plasma-cell hepatitis, lupoid hepatitis, and cirrhosis (Bjorneboe & Krajl 1977).

normal liver of rhesus monkeys as antigen. A positive complement fixation was also found in 18.8 per cent (24 out of 128) and in 14.1 per cent (19 out of 135) of the sera using normal kidney tissue and thyrotoxic thyroid glands, respectively as antigen.

Complement fixing liver antibodies were demonstrated in 33-38 per cent of the sera from patients with posthepatic and cryptogenic types of cirrhosis, in the alcoholic group a positive test occurred in 7 per cent, a frequency which did not differ essentially from that previously demonstrated in a material of normal blood bank donors.

The complement fixing liver antibodies were non species and non-organ specific. The 'pattern' of organ reactivity was rather constant for a given patient but varied apparently quite arbitrarily from one patient to the other.

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EXPERIMENTAL STREPTOCOCCAL NEPHRITIS IN RABBITS

By

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Early in this century a relationship between streptococcal infections and acute glomerulonephritis was considered (Ophule 1936). In 1952 Hammelkamp *et al.* and in 1953 Hammelkamp Hammelkamp *et al.* and Wertheim *et al.* presented epidemiologic evidence for a correlation between certain streptococcal types and nephritis and they suggested the term nephritogenic streptococci for these types. Type 12 streptococci appear to be the most potent etiologic agent in the development of acute glomerulonephritis in humans.

Experimental production of a typical acute glomerulonephritis in animals using β hemolytic streptococci or preparations thereof has not been successful (Horn 1937, Kolertuck 1952, Tan 1964). Although hematuria and sometimes albuminuria and hypertension have been induced in such experiments the histopathologic lesions have always been predominantly of tubular nature. Thus Reed & Matheson (1947a and b) were able to produce a clinical picture of acute nephritis in rabbits using type 12 streptococci; however the disease was largely devoid of the glomerular changes characteristic for acute glomerulonephritis in humans. Similar results have been published by Kelly & Winn (1958), Hinkle *et al.* (1960) and Tan & Kaplan (1962) using different types of intraperitoneal diffusion chambers containing cultures of β hemolytic streptococci. The chief barrier in experimental reproduction of acute glomerulonephritis appears to be the so-called nephrotoxic nephritis which may be prevented by injection of anti-kidney serum. In such experiments it has been found by Matsugi (1933-34) severe glomerular lesions with cellular infiltration and crescent formation could be produced.

Recent preliminary reports by Hille & Jansson (1964) and Jansson & Holm (1964) showed that it is possible by injecting different types of preparations from type 12 streptococci to induce histopathologic lesions

We wish to thank Prof. G. O. Osterborg for his stimulating interest and valuable criticism. The skill of technical assistance of Miss Harriet Andersson and Miss Brit Marie W. Westerberg is highly appreciated.

well as clinical signs of glomerulonephritis in rabbits. The present report comprises more detailed information on the preparation of the material and the development of the histopathological kidney changes.

MATERIALS AND METHODS

The nephritogenic streptococcal strain used was isolated from a patient with acute glomerulonephritis during an outbreak of streptococcal throat infections in Bedale, England, 1963. The strain was an group A type 12 (M+) streptococcus obtained from the Central Public Health Laboratory, Colindale, London.

Cultivations. The streptococci were grown in an antigen free medium described by Holm & Falsen (1966). The cultivations were performed at 37° C using an automatic titrator for pH adjustment (pH 6.8) and the cells were harvested after sixteen hours. The cultures were centrifuged at 1500×g for 20 minutes at 4° C. Three washings of the cells with saline buffered with phosphate M/15 pH 7.2 were performed under the same experimental conditions within one hour. Five volumes of the wet bacterial sediment were suspended in 95 volumes of buffered saline and the suspension was kept at 4° C for two weeks. It was shaken once or twice daily and finally filtered through a Göttingen No. 6 membrane filter. The filtrate was tested for sterility. This preparation was designated streptococcal 'autolysate' and was found to contain 280 γ protein/ml as measured by the method described by Lowry *et al.* (1951) using lysozyme as a reference substance.

Animals. 20 rabbits weighing between 2.5 and 3.0 kg were injected once intravenously with 0.5 ml of type 12 streptococcal 'autolysate'. The rabbits were sacrificed at different times after the injection (2, 4, 6 and 8 hours and 1, 3, 6, 13, 20 and 30 days). A control group consisting of two rabbits was injected once intravenously with 0.5 ml saline. All rabbits which were kept in the same animal room in separately ventilated animal boxes were obtained from the same farm.

Rabbit sera. Bleedings of all rabbits were performed immediately before the injection and at the time of sacrifice.

Rabbit kidney antigen. Kidneys from healthy rabbits were sliced and washed in phosphate buffered saline pH 7.2. Thirty ml buffered saline were added to 10 g of the rabbit kidney preparation and homogenized in an Ultra Turrax (Janke & Kunkel, H.G. Staufen i. Br.) at 24000 rpm for 3 minutes during cooling in an ice bath. The homogenized preparation was then freeze pressed 5 times in an X press (AB Blox, Nacka, Sweden). This material was centrifuged at 10000×g for 20 minutes. The supernate was pipetted off and frozen at -40° C until used. The protein content of this preparation was 10 mg/ml as measured by the method of Lowry *et al.* (1951) using lysozyme as a reference.

Diffusion in gel method. The rabbit sera were analysed by the double diffusion in gel method of Ouchterlony (1938) as modified by Holm (1965) against different concentrations of the rabbit kidney antigen (0.01–10 mg/ml).

Complement fixation method. The complement fixation method was performed according to the description by Kabat & Mayer (1964). Heat inactivated rabbit kidney antigen was used in the highest concentration that gave a positive anti-complementary effect and analysed against the rabbit sera in different dilutions (1/1–1/320).

DNase activity. was determined according to the technique of Carlson *et al.* (1957). Determinations of DNase inhibitors as well as DNase antibodies were performed according to a technique described by Holm & Kaijser (1967).

Streptolysin O (SLO) activity in streptococcal preparations was determined according to Kalbak (1947) and expressed in combining units using a standard anti-streptolysin from Statens Seruminstitut, Copenhagen.

Antistreptolysin II determinations were performed according to the technique of Kalbak (1947).

Streptolysin S (SSS) activity was determined by measuring the hemolytic activity on a 2 per cent suspension of rabbit erythrocytes. Minute amounts of cholesterol were added to inhibit the hemolytic activity of SLO according to Houard *et al.* (1953).

Streptococcal proteolase activity was measured by the milk clotting method described by Ellis et al (1943).

Blood pressure readings. The method used for measuring blood pressure in rabbits was a modification of the method described by Grant & Rotschill (1924). A lens (magnification 25 X) was inserted with an air tight seal in the upper end of a metal tube and at the lower end a piece of cnd m rubber was fixed. This sealed system was mounted on a microscope stand and connected to a plastic tube to a mercury manometer and by a T tube a sphygmomanometer bulb. The rabbit's ear was placed between the cuff and membrane and a glass slide attached to the stage of the microscope light from a microscope lamp was directed through the glass slide by increasing the air pressure the flat end of the tube and compressed the artery. The pressure readings were taken when the first sign of blood reappeared in the artery. Ten consecutive readings were performed and the average value determined (limit values ± 4 mm Hg from the average value). The blood pressure in the ear artery of untreated rabbits varied between 20-30 mm Hg.

Proteinuria and hematuria. Determination of proteinuria was performed semi quantitatively by the albumin method (Ames Co., St. Louis, England) and when positive further confirmed with the sulfosalicylic acid method (Kingsbury et al 1976). The occult test method (Ames Co., St. Louis, England) was used as a screening test for hematuria. In case of positive outcome the urine cast was examined microscopically in a Fuchs-Rosenthal chamber.

Histopathological examination. At different intervals after the injection two rabbits at a time were sacrificed by bleeding. The kidneys were prepared for the histopathological examination by cutting 1 mm thick slices which were fixed in Zenker solution. The pieces were embedded in paraffin and after sectioning various staining methods were applied such as hematoxylin-eosin, hematoxylin-van Gieson, Papanicolaou and finally in some cases the Weiler silver impregnation method. At least three sections from each kidney from every rabbit were examined and only definite repeatedly found histological changes were registered.

RESULTS

Enzyme activity in different streptococcal preparations. Culture filtrate from the type 12 strain used was found to contain 15 combining units S/O and 1200 DPNase units per ml. Streptococcal proteinase was mostly present in the precursor form and even so in small amounts. S/S activity was not detected in the culture filtrate. Neither S/O, S/S, DPNase nor proteinase was found in the "autolysate" preparation but after concentration some DPNase inhibitory effect was noted corresponding to 20-30 inhibitor units per ml unconcentrated "autolysate" material. Antibodies to these streptococcal products could not be detected in any of the rabbits.

Blood pressure readings. After the injection blood pressure readings were performed at different intervals. From Figs. 1 and 2 it is evident that rabbits injected with "autolysate" from the type 12 (M+) streptococcus developed an extreme hypertension. This started almost immediately after the injection and reached a level as high as 3-4 times the initial value (90 mm Hg) during the following 9 hours. The blood pressure attained its maximum (130 mm Hg) after 4-5 days. Two months after the injection the blood pressure was still high but had decreased to 70-80 mm Hg.

Proteinuria and hematuria. Proteinuria was found in one of the two rabbits sacrificed one day after the injection while the second as well as two other rabbits sacrificed two days later showed no proteinuria.

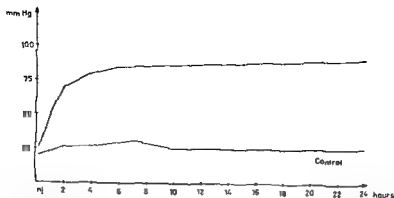


Fig 1

Average blood pressure in the ear artery of twelve rabbits injected with the streptococcal "autolysate" preparation from a type 12 streptococcus (measured by the ear capsule method)

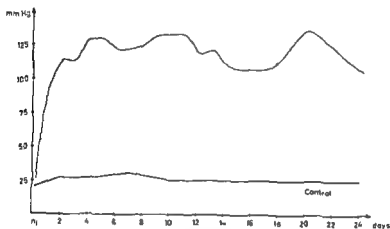


Fig 2

Average blood pressure in the ear artery of four rabbits injected with the streptococcal "autolysate" preparation from a type 12 streptococcus

One of the rabbits, killed 6 days after the injection, as opposed to all of those killed after 13 days, showed the presence of protein in the urine. The rabbits sacrificed at later stages had traces to moderate amounts of protein in the urine. Hematuria was also occasionally found during the first six days after the injection. At later stages half the number of animals developed hematuria, which was often only detected by microscopic examination.

Antibodies against homologous kidney tissue Although three different serological techniques were employed, antibodies against homologous kidney tissue could not be revealed in the sera from any of the rabbits irregardless of the severity of the kidney lesion produced. Not even those rabbits which were sacrificed two months after the in-

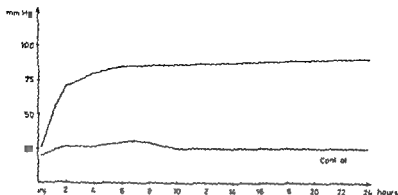


Fig. 1

Average blood pressure in the ear artery of twelve rabbits injected with the streptococcal autolysate preparation from a type 12 streptococcus (measured by the ear capsule method)

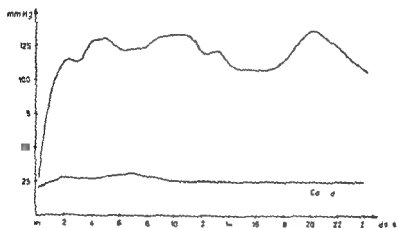
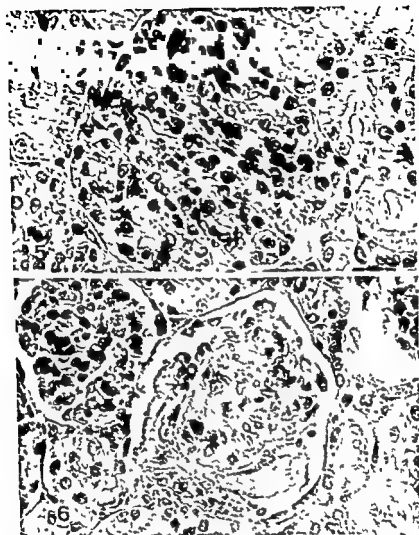


Fig. 2

Average blood pressure in the ear artery of four rabbits injected with the streptococcal "autolysate" preparation from a type 12 streptococcus

One of the rabbits killed 6 days after the injection as opposed to all of those killed after 13 days showed the presence of protein in the urine. The rabbits sacrificed at later stages had traces to moderate amounts of protein in the urine. Hematuria was also occasionally found during the first six days after the injection. At later stages half the number of animals developed hematuria which was often only detected by microscopic examination.

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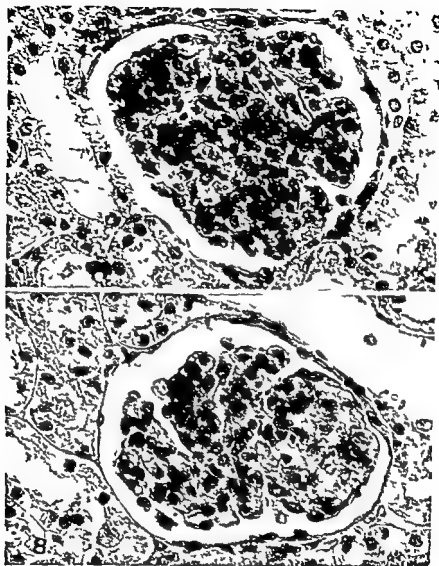


Figs 5-6

Fig 5 Enlarged glomerulus with swollen and proliferating endothelial cells. Hematoxylin & Eosin $\times 400$

Fig 6 Two glomeruli one only slightly swollen and with proliferating capillary endothelium the other with advanced changes among other things a fibrinous exudate in the capsular space. Hematoxylin & Eosin $\times 400$

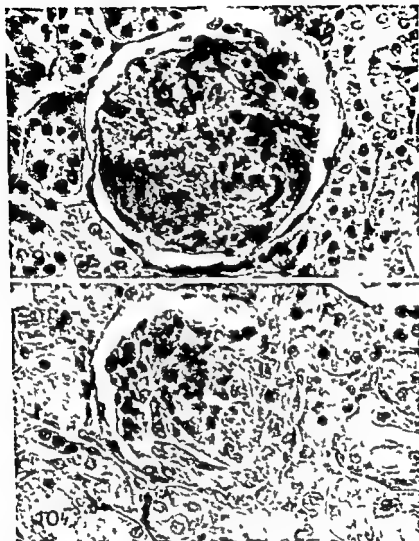
twelve rabbits one (No 3) showed no glomerular changes and one rabbit only an intense hyperemia (No 1). In two animals (Nos 2 and 9) the main glomerular change was a pronounced swelling of the capillary endothelium. In three animals (Nos 4, 11 and 12) this swelling was combined with a proliferation of the endothelial cells, especially in rabbit No 6. Even at low magnification the hypercellularity of the damaged glomerulus was striking (Fig 3). In one rabbit (No 10) the



Figs 7 & 8

- Fig 7* Glomerulus with very enlarged endothelium proliferation of the endothelium and fusion of the capillary loops Hematoxylin & Eosin $\times 400$
- Fig 8* Glomerulus with swollen and proliferating capillary endothelium finger like capillary loops and finger like apposition Hematoxylin & Eosin $\times 400$

capillary loops were fused together and besides the endothelial swelling a proliferation of the capsular epithelium was noted in some glomeruli. In three animals (Nos. 5, 7 and 11) advanced lesions were seen affecting practically all glomeruli. Besides swelling and proliferation of the capillary endothelium (Fig. 7) fibrinous exudation in the capsular space (Fig. 6) and a finger like fusion of the capillary loops could be seen (Figs. 7 and 8). In two of these animals a hemorrhagic necrosis



Figs 9-10

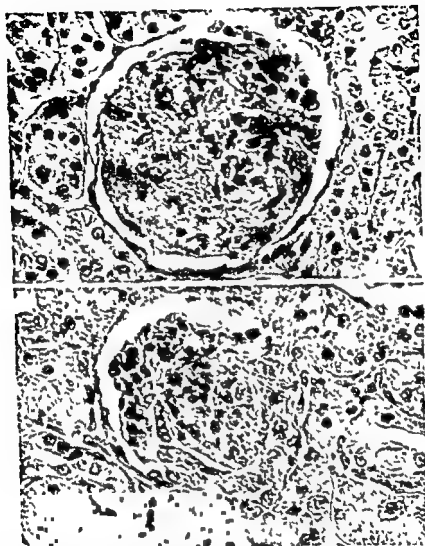
Fig 9 Partially hemorrhagic necrotic glomerulus. Hematoxylin & Eosin $\times 400$

Fig 10 Advanced changes of the glomerulus with sickle-like proliferation of the epithelium of Bowman's capsule. Hematoxylin & Eosin $\times 400$

of some glomeruli was also detected (Fig. 9). In the third rabbit (No. 7) a proliferation of the epithelium in Bowman's capsule with development of adhesions between the sheaths of the capsule was found (Fig. 10). In silver stained sections a network of argyrophilic threads could be seen in the changed glomeruli.

All rabbits with advanced glomerular changes also presented tubular lesions. These were characterized by swelling of the epithelium in the tubuli contorti in combination with a decreased affinity for nuclei

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Figs. 9-10

Fig. 9 Partially hemorrhagic necrotic glomerulus. Hematoxylin & Giesson $\times 400$

Fig. 10 Advanced changes of the glomerulus with sickle like proliferation of the epithelium of Bowman's capsule. Hematoxylin & Giesson $\times 400$

of some glomeruli was also detected (Fig. 9). In the third rabbit (No. 7) a proliferation of the epithelium in Bowman's capsule with development of adhesions between the sheets of the capsule was found (Fig. 10). In silver stained sections a net of granular argentophilic threads could be seen in the changed glomeruli.

All rabbits which showed glomerular changes also presented tubular lesions. These were seen as gentle swellings of the epithelium in the tubuli contorti in combination with a decreased affinity for nuclear

stains. Red blood corpuscles and hyaline and/or granular cylinders especially in the collecting tubes were also noted (Fig. 4). In one rabbit (No. 3) without glomerular changes, red blood corpuscles were seen in the collecting tubes. The severity of the tubular changes was on the whole proportional to the glomerular changes.

No histopathological glomerular or tubular changes could be detected in the control rabbits.

DISCUSSION

In the preliminary reports on the nephritogenicity of streptococci (Holm & Jonsson (1964), Jonsson & Holm (1964)) it was shown that acute glomerulonephritis could be produced by culture filtrates as well as "autolysate" preparations or penicillin-killed type 12 streptococci. These results disagree with the findings of Reed & Mathison (1954 b), who could not produce hypertension or any kidney lesions by injections of ten-times washed and killed streptococci. This discrepancy can be explained if it is assumed that the nephritogenic factor or factors diffused from the streptococci into the washings and were thus removed from these streptococcal preparations. Tan & Kaplan (1962) reported that tubular lesions in mice could not be produced by streptococcal (Group A) culture filtrates lacking streptolysin S (SIS) activity and they could not protect the animals against the tubular toxic effect by injection of polyvalent streptococcal antisera. On this basis they have suggested SIS as a possible agent responsible for the tubular lesions in acute glomerulonephritis in humans. In our "autolysate" preparations no SIS activity was found which may be due to unfavourable conditions for preserving SIS activity during the preparation of the material. As was shown in an earlier publication (Jonsson & Holm 1964) an "autolysate" preparation of the streptococcal strain S 84 (Group A, type 3) as well as a similar preparation of a strain of *Streptococcus fecalis* hemolyticus (Group D) could produce tubular lesions in rabbits but neither glomerular lesions nor hypertension were produced. SIS need not therefore be the only nephritogenic agent in acute glomerulonephritis.

Furthermore another well-known streptococcal product—streptococcal DPNase—has been considered as a possible etiologic factor in the development of acute glomerulonephritis (Rammelkamp 1957, Bernheimer *et al* 1957). In our studies, however we could find no DPNase activity in the autolysate preparation although the corresponding culture filtrate contained 1200 DPNase units/ml. The newly described (Holm & Kayser 1966) DPNase inhibitor is present in fairly high amounts and the presence of an inhibited DPNase could therefore not be ruled out. On the other hand the strain S 84, as well as many other group A streptococcal strains of different serological types, produce DPNase (Holm & Kayser 1966) which contradicts DPNase as an important nephrotoxic factor, at least in regard to glomerular lesions. Furthermore neither SIO nor streptococcal proteinase activity could

be related to nephritogenicity as none of these enzymes could be detected in the autolytic preparation. Nor did the rabbits produce detectable amounts of antibodies against streptolysin O, proteinase or DPNase.

Hypertension was produced within a few minutes after the injection of streptococcal "autolytic" before any demonstrable microscopic glomerular or tubular lesions. The interpretation of these blood pressure findings which were reproducible in all rabbits is difficult. It is of course possible to assume a rapidly progressing kidney damage at this early stage. That at least glomerular changes may develop early is indicated by an extreme hyaline in the glomeruli of the rabbits sacrificed within 24 hours after the injection. If a glomerular lesion develops immediately after the injection, an etiologically important factor would be an ischemia of the kidney. The hypertension could then be comparable to the *Goldblatt* mechanism (1917) with release of renin. It is also possible that the nephritogenic streptococci themselves contain substances which initiate an ischemia of the kidney with secondary release of vaso-constrictor material from the kidney. The persistence of the hypertension indicates that a hypertensive factor in nephritogenic streptococci is probably not the *only* etiological agent to the hypertension. It is noteworthy that no latent phase of the development of hypertension was seen after the injection of streptococcal material. The possibility of an antigen-antibody reaction is an explanation to the hypertension can of course not be completely ruled out by the experiments performed but the early onset of hypertension would be difficult to explain on immunological bases.

The results of the determinations of hematuria and proteinuria confirm the clinical diagnosis of acute glomerulonephritis. As is also often found in humans, albuminuria was occasionally found in early stages of acute glomerulonephritis. However, in rabbits with advanced histopathological changes (2-3 weeks after the injection) proteinuria was almost always found and often of marked severity. Hematuria was also detected in most rabbits. In fact hematuria seemed to be an early sign of nephritis, since blood in the urine was found in some rabbits already the day after the injection. In one of these cases red blood corpuscles and granular cylinders were also found in the tubuli at the histopathological examination. In the tubuli of rabbits sacrificed at later stages erythrocytes, hyaline and/or granular cylinders were an almost regular finding.

The kidney changes observed microscopically correspond essentially to those seen in the acute and subacute stages of glomerulonephritis in humans. However, it was noted that only some of the glomeruli were affected when the pronounced renal changes were produced. Furthermore, in some animals lesions were also found corresponding largely to focal glomerulonephritis in humans.

In spite of the established hypertension no hypertensive changes of

the vessels could be demonstrated in the kidneys, which may be due to the short duration of the hypertension

In review articles *Rother & Sarre* (1961), *Cruickshank* (1962), *Dixon & Feldman* (1964) and *Paunz* (1965) discussed different pathogenetic mechanisms in the induction of experimental glomerulonephritis. That anti-kidney antibodies can be detected in humans with streptococcal nephritis has been reported by many authors (*e.g.* *Lange et al* 1949, *Liu & McLeroy* 1958) but the significance of such findings is debatable in spite of the demonstration of host γ -globulin (*Mellors et al* 1957) and C' (*Luchmann et al* 1962) in glomeruli of patients with glomerulonephritis. In our experiments no anti-kidney or anti-streptococcal antibodies could be detected in the sera of the diseased rabbits by any of the three immunological techniques used. It therefore seems probable that the kidney lesions produced were the results of a direct toxic effect induced by one or more streptococcal factors rather than an anti-kidney antibody reaction. However, we cannot rule out the possibility that an immunological mechanism may also be involved. This would then be elicited by a mechanism analogous to the one described by *Kay* (1940) due to fixation of streptococcal antigens to the kidney just as streptococcal M-protein has been found by *Kaplan* (1958) to have an unusual affinity to the glomeruli of the kidney.

SUMMARY

Type 12 streptococci isolated from a patient with acute glomerulonephritis, were grown in an antigen free medium under stabilized pH conditions. The cells were washed and "autolyzed" by storing at 4°C for 2 weeks. After sterile filtration the "autolysate" preparation was injected intravenously into rabbits. Clinical and histopathological evidence of the production of glomerulonephritis was obtained. No kidney antibodies could be detected in the sera from any of the rabbits.

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EXPERIMENTAL AND RESULTS

Hydrolysis of the Acidic Polysaccharide

The monosaccharide components were determined after hydrolysis with sulphuric acid. The hydrolysate was neutralized with barium carbonate and centrifuged. The supernatant was treated with Amberlite Resin IR 120 (H) and concentrated to a small volume ready for paper chromatography. Descending chromatography on Whatman paper no. 1 was used. Solvent developers were

- 1 Butanol-ethanol-water 4:1:5
- 2 Ethyl acetate-pyridine-water 5:2:5
- 3 Pyridine-ethyl acetate-glacial acetic acid-water 5:5:1:3
- 4 Butanol-ethanol-water-ammonia 40:10:40:1

Most of the chromatograms were run twofold for spraying with the two different reagents a) aniline hydrogen phthalate and b) silver nitrate-sodium hydroxide.

Hydrolysis with Different Concentration of Sulphuric Acid

The same amount of polysaccharide (10 mg per ml of acid) was hydrolysed for 3 hr at 100° with different concentration of sulphuric acid 2N, 1N, 0.1N and 0.01N. The hydrolysates were neutralized in the usual way and submitted to paper chromatography in solvent 1 and 2. The hydrolysates were found to contain

2N sulphuric acid glucose, mannose, uronic acid and aldohuronic acid

1N sulphuric acid glucose, mannose, uronic acid and aldohuronic acid but also a fraction with R_f value between those for uronic acid and glucose

0.1N sulphuric acid gave the same fractions as hydrolysis with 1N sulphuric acid but on a smaller scale

With 0.01N acid most of the polysaccharide was still unhydrolysed but a faint spot of mannose could be seen

When the polysaccharide was hydrolysed with 2N, 1N or 0.1N sulphuric acid and the paper chromatogram of the hydrolysates was sprayed with silver nitrate-sodium hydroxide a spot appeared which could not be seen when sprayed with aniline hydrogen phthalate. The R_f value corresponded with the lactone spot of glucuronic acid.

The results from the different hydrolyses showed that the polysaccharide was composed of glucose, uronic acid which easily formed lactone, a small amount of mannose and aldohuronic acid.

Cellulose Column Chromatography of the Hydrolysate of the Polysaccharide

The preliminary hydrolysis indicated that 1N sulphuric acid probably would give a disaccharide different from aldohuronic acid. This

concentration was therefore used when a big portion of the polysaccharide was hydrolysed for column chromatography.

400 mg of acidic polysaccharide was hydrolysed with 1N sulphuric acid for 3 hr at 100°. The hydrolysate was treated in the usual way before evaporation to dryness to obtain the weight which was 300 mg. The column was packed with Whatman standard grade cellulose powder as a slurry in solvent no. 1 which was used as developer during the chromatography. The hydrolysate was applied to the top of the column dissolved in solvent no. 1. Fractions of 5 ml were collected by an automatic fraction collector at a rate of three fractions an hour. Spots of each second fraction were applied to a paper chromatogram in solvent no. 2 and sprayed with silver nitrate sodium hydroxide. Five different fractions were collected from the column and identified by paper chromatography as glucose, mannose, uronic acid, aldohuronic acid and another disaccharide with R_F value between those for glucose and uronic acid.

The glucose fraction showed an optical rotation $[\alpha]_D^{20} = +44^\circ$ (c 0.4 water). The glucose was further identified by treatment with glucose oxidase. Gluconic acid was formed which could be seen when the paper chromatogram was sprayed with silver nitrate sodium hydroxide.

Mannose was only identified on paper chromatograms in solvent no. 1, 2 and 3 since the amount isolated was too small to make any derivatives.

The uronic acid was identified as glucuronic acid by conversion to the methyl ester methyl glycoside reduction with lithium aluminium hydride, hydrolysis and paper chromatography of the hydrolysate. This contained only glucose which was further identified by glucose oxidase.

The aldohuronic acid fraction was hydrolysed with 2N sulphuric acid for 8 hr and was found to contain glucose and uronic acid; the disaccharide was converted to the methyl ester methyl glycoside and reduced with lithium aluminium hydride. The hydrolysate of the methyl bioside contained glucose only which was further identified by glucose oxidase. The aldohuronic acid was therefore identified as glucuronic acid linked to glucose.

The other disaccharide also had a R_F value between those for glucose and glucuronic acid; it is hydrolysed and was found to be composed of mannose and glucose.

Periodate Oxidation of the Acidic Polysaccharide

The acidic polysaccharide was oxidized by sodium periodate at room temperature for 100 hr. The consumption of periodate was analysed periodically. The results are plotted against time and can be seen in Table 1. It was found that oxidation was complete after 48 hr and

overoxidation started. This was confirmed when the oxidation was repeated at 4°.

To see if acid was formed during oxidation, aliquots of the reaction mixture were analysed. Excess of periodate was removed by addition of ethylene glycol, and acid formed during the oxidation was titrated against 0.01N sodium hydroxide potentiometrically by means of a glass electrode and a pH-meter. The results can be seen in Table 1.

TABLE 1
Periodate Oxidation of the Polysaccharide

Time in hr	1	4	24	48	72	120	144
Mol periodate per mol anhydrosugar	0.5	0.56	0.91	1.04	1.21	1.40	1.38
Mol formic acid formed per mol anhydrosugar	-	-	0.30	0.46	0.54	0.54	-

Any measurable amount of formaldehyde was not formed during the oxidation with periodate.

The polysaccharide was isolated after oxidation and hydrolysed. By paper chromatography it was found to contain mostly glucose. Only traces of mannose and glucuronic acid were left.

Methylation of the Acidic Polysaccharide

Haworth's method was used for methylation. The procedure was repeated four times. After isolation, the methylated polysaccharide was hydrolysed in two stages, first by treatment with 5 per cent methanolic hydrogen chloride and then with 2N sulphuric acid. The hydrolysate was applied to a paper chromatogram in solvent no. 4. The chromatogram was stopped before the solvent front left the paper, about 17 hr. Six spots were seen when the paper was sprayed with anilin hydrog. phthalate. When the hydrolysate was deionized with De-acidite Resin FF (CO₂) before chromatography, two of the spots disappeared ($M_R = 0.16$ and 0.06) which indicated that these spots belonged to the methylated uronic acid(s). M_R -value for 2,3,4,6-tetra-O-methyl-glucose in the same solvent was 1.00.

By comparing ionophoresis in 0.1M borate buffer, pH = 10 and M_R values by paper chromatography, the neutral methylated monosaccharides have been identified as 2,3,4,6-tetra-O-methyl-mannose, 2,4,6-tri-O-methyl-glucose and 2,3-di-O-methyl-glucose. One of the spots could not be identified, and could well be due to incompletely methylated sugar.

The methylated glucuronic acid was removed from the hydrolysate by De-acidite Resin FF (CO₂). The resin was treated with ammonium carbonate, to get the acid out again as ammonium salt. The solution

with the ammonium salt was treated with Amberlite Resin 1 B 120 (H) to obtain the methylated uronic acid as free acid. The acid was then converted to methyl ester methyl glycoside, reduced with lithium aluminium hydride, hydrolysed, and the methylated glucose was identified as 2,3,4 tri O methyl glucose.

By comparing the results obtained after oxidation and after methylation the glucuronic acid could be linked 1-6. Most of the glucose seemed to be linked 1-3, but some glucose is engaged in branching as 1-4-6 linked.

The mannose is linked to glucose either in 1-6- or in 1-4 position and will probably form the non reducing end group at the branch point.

DISCUSSION

The first investigation of the capsular polysaccharide isolated from *Klebsiella pneumoniae* type 2 (B) showed it to contain glucose and uronic acid. Later the presence of rhamnose and the nature of the uronic acid as glucuronic acid were determined. This very clearly explained the cross reaction between *Klebsiella pneumoniae* type 2 (B) and *Pneumococcus* type 2, since both the type specific polysaccharides were composed of identical monosaccharides.

However, in the examination of the acidic type specific polysaccharide from *Klebsiella pneumoniae* type 2 (B) which has been referred to in this paper, rhamnose could not be demonstrated. Rhamnose was not found neither in the crude polysaccharide nor in the purified acidic polysaccharide. Instead of rhamnose, mannose was found. This monosaccharide has not been reported as constituent of the polysaccharide from *Klebsiella pneumoniae* type 2 (B) before. The amount of mannose was very small, in fact too small to isolate for making derivative. The presence of mannose has therefore only been ascertained by paper chromatography, but in three different solvent developers.

The mannose seemed to be the non reducing end group at the branch point. The presence of 2,3,4,6-tetra O methyl mannose after methylation and hydrolysis confirmed mannose 1-1 the non reducing end group. Mannose was also preferentially released on treatment of the polysaccharide with 0.01N sulphuric acid for 3 hr at 100°.

The polysaccharide cannot have a highly branched structure, since oxidation with periodate did not form any measurable amount of formaldehyde.

Glucose seemed to form the branch point which was proved by identification of 2,3,4 tri O methyl glucosides. The isolation of a small amount of the disaccharide composed of glucose and mannose confirmed glucose as branch point.

Most of the glucose, however, must be a part of the chain structure and linked 1-3. After methylation and hydrolysis of the polysaccharide most of the glucosides was identified as 2,4,6 tri O methyl glucose. When

the polysaccharide was isolated and hydrolysed after oxidation, glucose was found in a big yield

The glucuronic acid seemed to be linked 1,6. By oxidation formic acid was formed. The identification of 2,3,4-tri-O-methyl-glucose after reduction of the methylated uronic acid fraction, confirmed the presence of 1,6-linked glucuronic acid. The uronic acid is linked to glucose as an aldohuronic acid.

The glucuronic acid linked 1,6 could be the main common feature in the two type specific polysaccharides isolated from *Klebsiella pneumoniae* type 2 (B) and *Pneumococcus* type 2, and make the foundation for the cross-reaction between the two species. Another point could be the presence of glucose as branch point in both polysaccharides.

SUMMARY

- 1 The acidic type specific polysaccharide of *Klebsiella pneumoniae* type B (B) was isolated and further purified for structure analysis.
- 2 The polysaccharide contained glucose and glucuronic acid, but also a small amount of mannose was found.
- 3 The glucuronic acid was linked to glucose as an aldohuronic acid.
- 4 The glucuronic acid was linked 1,6.
- 5 Most of the glucose was linked 1,3, but some of the glucose formed branch points in 4- or 6-position.
- 6 Mannose could be the non-reducing end group at the branch point.

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SPECIFICITY OF RHEUMATOID FACTORS IN SERA FROM BLOOD DONORS

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The rheumatoid factors are usually γ M globulins and behave as antibodies to determinants of the Fc fragment of the γ G globulin molecule (11). This antibody property is utilized for their detection. In the most frequently employed tests for rheumatoid factors, erythrocytes sensitized by human (isologous) or rabbit (heterologous) γ G globulin are used (see 11 and 25). The rheumatoid factors are inhibited by whole γ globulin in native or denatured form.

Rheumatoid factors which detect genetic characters (antigen factors) are most often found in sera from patients with rheumatoid arthritis. They may also result from transfusion (2, 22) or foeto-maternal (5) or materno-foetal (23, 24) immunization. Some sera from healthy individuals without known immunization may contain these types of rheumatoid factors.

Rheumatoid factors which do not detect genetic characters are also found in sera from certain patients and from some healthy individuals. Earlier results showed that in many sera from patients with rheumatoid arthritis the reactivity of these factors was caused by separate molecules with specificity for either human or rabbit γ globulins, besides cross reacting factors (14, 15, 26, 21).

In the present work, a large panel of blood donor sera was tested for rheumatoid factor using rabbit γ globulin (Wickler-Rose test). The reactivity of some of the positive sera with human γ globulin was investigated and the specificity of the factors was compared to that described for sera from patients with rheumatoid arthritis. The incidence of positive reactions in the normal population was not considered since it has been extensively discussed in earlier literature (see 28).

MATERIALS AND METHODS

Sera. Sera from 2245 blood donors were obtained from the Serological Laboratory, Department of Microbiology, the University of Bergen. They had been sent for routine

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syphilis serology The blood donors had passed an ordinary clinical examination and showed no signs of disease. Some rheumatoid sera used in a previous study (26) were included.

The incomplete anti D sera, the reference anti Gm sera and the rabbit anti human γ globulin serum and specific anti γ G, anti γ A and anti γ M sera were the same as used previously (16). Anti γ A and anti γ M sera were purchased from Behringwerke AG Marburg-Lahn, Germany. Incomplete anti CD serum Ripley was kindly provided by Dr Marion Waller of the Department of Pathology, School of Medicine, Richmond, Va., USA.

Rabbit anti sheep erythrocytes serum was prepared as described earlier (25).

The sera were kept frozen at -20°C in small aliquots. All sera except the anti D sera were heated for 30 minutes at 56°C before use.

Treatment of sera by 2 Mercaptoethanol was performed as described previously (27).

Gamma globulin Human γ globulin (Fraction II), 12 per cent solution was purchased from AB Kabi, Stockholm, Sweden. Rabbit γ globulin (lyophilized Fraction II, a preparation of Pentex Inc., Kanakakee, Ill.) was a gift from Dr F. Milgram, Buffalo, N.Y. The protein concentration was determined by a modified Folin method (13) using the γ globulin from AB Kabi as standard.

Denatured γ globulin preparations were prepared by heating a 1 per cent solution at 63°C for 10 minutes.

Erythrocytes Human erythrocytes (O, R_1R_2) from a single donor were collected in sterile acid citrate dextrose solution. Sheep erythrocytes were obtained from whole blood collected in Alsever's solution. Before use the erythrocytes were washed 4 times in 10 volumes of saline (phosphate buffered saline pH 7.2). They were finally packed at $1000 \times g$ for 5 minutes.

Tests for anti γ globulin factors The Waaler Rose test was carried out as described elsewhere (25, 26). After the tubes had stood overnight at 4°C the agglutination was recorded by gently tapping each tube.

The tests with anti D sensitized erythrocytes were performed as previously described, using an excess of incomplete anti D antibody for sensitization (16). The sensitized erythrocytes were always tested with rabbit anti γ globulin serum and reference anti Gm sera before use. One drop each of serum dilution, saline and a one per cent suspension of sensitized erythrocytes were mixed and left at 20°C for 1 hour. Before reading the tubes were centrifuged at $1000 \times g$ for 30 seconds.

The latex (RA) test was performed as recommended by the manufacturer with reagents purchased from the Hyland Laboratories, Los Angeles, Calif., USA.

Inhibition of agglutination The γ globulin preparations were properly absorbed by unsensitized or by sensitized erythrocytes in order to remove agglutinating activities against unsensitized and sensitized erythrocytes. Absorbed γ globulin was tested for inhibiting activity by adding 0.1 ml of a 0.5 per cent solution to 0.1 ml of twofold dilutions of the serum to be tested. After incubation for 1 hour at 20°C the sensitized erythrocytes were added and agglutination was recorded in the usual manner.

Absorption of anti γ globulin factors Absorption using human γ globulin was performed by mixing 0.2 ml of serum dilution 1 in 2 and an equal volume of packed erythrocytes sensitized by anti CD Ripley III excess. The mixture was left at 20°C for two hours. The supernate (absorbed serum) was removed after centrifugation.

Absorption using rabbit γ globulin was performed by mixing 0.2 ml of serum diluted 1 in 8 and 1 packed sheep erythrocytes sensitized by ten agglutinating units of rabbit antiserum. The mixture was left at 4°C overnight. The absorbed serum was tested against unsensitized and sensitized sheep erythrocytes.

Preparative ultracentrifugation Preparative ultracentrifugation was performed according to the method described by Kunkel *et al.* (10) using 16-hour separation in a 10-40 per cent sucrose gradient. Ten successive 0.5 ml fractions of the effluent were obtained from a pin hole in the bottom of the tubes.

EXPERIMENTS AND RESULTS

Agglutination Experiments

Of the 2295 blood donor sera 2137 (93.5 per cent) gave titres of 16 or less in the Waler Rose test (Table 1). Such weak reactions were considered as negative. Titres of 32 or 64 were obtained with 130 of the sera (5.7 per cent), and were regarded as doubtful positive reactions. Titres of 128 or higher obtained with 18 sera (0.8 per cent), were considered as definite positive reactions. The latter group comprised an equal number of males and females aged between 20 and 55.

Because of the small amounts of each serum available only 14 sera were further studied. Twelve of these were selected among those which gave highest titres in the Waler Rose test. Two sera which gave no reaction in the Waler Rose test were also included.

None of the sera agglutinated erythrocytes sensitized with anti D specific for Gm(1), Gm(4) or Gm(5) respectively. The results obtained in the anti CD Ripley test and in the latex test are shown in Table 2. The latex test positive sera gave either positive or negative reactions in the anti CD Ripley test while all the latex test negative sera gave no reaction in the anti CD Ripley test. One of the Waler Rose test negative sera (No. 1010) was positive in the anti CD Ripley test and the latex test.

TABLE 1
Distribution of the 2295 Blood Donor Sera According to Titres in the Waler Rose Test

	Titre in Waler Rose test					Total
	≤ 16	32	64	128	256	
Number of sera	2137	89	49	13	3	2295
Per cent of sera	93.5	5.7		0.8		100

TABLE 2

Fourteen Selected Blood Donor Sera Grouped According to Titres in the Waler Rose Test and to Results with the Latex and the Anti CD Ripley Tests

		Titre in Waler Rose test			Total
		≤ 16	32-64	≥ 128	
Latex test positive	Anti CD Ripley test positive (titre = 4)	1/10	865/991	8/579	5
	Anti CD Ripley test negative (titre < 4)		819/972	267/470/536	5
Latex test negative	Anti CD Ripley test positive				0
	Anti CD Ripley test negative	4/9	760	2/6/563	4

Absorption Experiments

The specificities of the anti- γ -globulin factors involved in the reaction with erythrocytes sensitized by human and rabbit antibodies were tested in absorption experiments with six of the sera. The results with two blood donor sera, and one rheumatoid arthritis serum included as control, are shown in Table 3. Sheep erythrocytes sensitized with rabbit γ -globulin easily removed the homologous activity, while the activity detected by the other test system was hardly influenced. Similarly, human erythrocytes sensitized with human γ -globulin removed only the homologous activity. This was found with the monospecific serum (849), showing reaction only in the Waaler-Rose test, as well as with the polyspecific serum (994) which reacted in both tests. Accordingly, anti- γ -globulin factors in sera from some blood donors exhibited a strict specificity, comparable to those in sera from patients with rheumatoid arthritis (14, 26). The other four sera contained mainly cross-reacting factors.

TABLE 3

Titres in Anti CD Ripley and Waaler Rose Tests of two Blood Donor Sera (NHS) and one Rheumatoid Serum (RAS) Effect of Absorption with Erythrocytes Sensitized by Rabbit and Human γ Globulin

Serum absorbed with	Anti CD Ripley test			Waaler-Rose test		
	NHS 994	NHS 849	RAS 4429	NHS 994	NHS 849	RAS 4429
<i>Sheep erythrocytes</i>						
sensitized	8	<2	32	<8	<8	8
unsensitized	8	<2	32	128	64	256
<i>Human erythrocytes</i>						
sensitized	<2	<2	<2	128	64	256
unsensitized	16	<2	32	128	64	256

Controls

Unsensitized erythrocytes and respective sera diluted 1 in 2: no agglutination

Sensitized erythrocytes and saline: no agglutination

Inhibition Experiments

The inhibiting effect of native and denatured human and rabbit γ -globulin on the agglutination in the Waaler-Rose and the anti-CD Ripley tests was investigated using the same sera as above.

Rabbit γ -globulin, either native or denatured, inhibited only the reaction in the Waaler-Rose test, while the reaction in the anti-CD Ripley test was not influenced (Table 4). Human γ -globulin inhibited completely the reaction in the anti-CD Ripley test, while the reaction in the Waaler-Rose test was only partially inhibited. Denatured human γ -globulin gave somewhat stronger inhibition than native γ -globulin.

With one blood donor serum, 849, which reacted in the Waaler-Rose test only there was no inhibition with either of the human γ -globulin preparations.

TABLE 4

Effect of Nature and Denaturation of Human or Rabbit γ Globulin on Titres of Immune Blood from Sera (NHS) and of Rheumatoid Serum (RAS)

Test in	Anti CD High test			Waller Rose test		
	NHS 994	NHS 819	RAS 4179	NHS 994	NHS 819	RAS 4179
Nature	16	2	32	124	124	24
γ -globulin (5 mg/ml)						
rabbit native	16	<2	32	<8	<8	<8
" denatured	8	<2	32	<8	<8	<8
human native	<2	<2	<2	32	64	32
" denatured	<2	<2	<2	16	64	16

Controls: Unsensitized erythrocytes and saline in agglutination
Sensitized erythrocytes and saline or the respective
 γ globulin preparations in agglutination

The findings were in accordance with those obtained in absorption experiments and with previous studies on rheumatoid factors in sera from patients with rheumatoid arthritis (11-26). Different specificities of rheumatoid factors in normal human sera seems to depend on separate factors with specificity for human or rabbit γ globulin or for both species γ globulins.

Physicochemical Properties

By preparative ultracentrifugation of three blood donor sera (570, 819, 914) the hetero- and isospecific rheumatoid factor activity was found in the bottom fractions. These fractions contained γ M globulin as shown in test with specific anti γ G, anti γ A and anti γ M sera. The rheumatoid factor activity was also inactivated by mercaptoethanol. Accordingly the serological activity was attributable to the γ M globulin.

DISCUSSION

Rheumatoid factors which detect genetic characters show a high degree of specificity (11). More than 20 such anti Gm factors are known at present (12-18, 21). It is also known that anti Gm factors from normal individuals (S₁g₁ factors) show a higher degree of specificity than anti Gm factors from patients with rheumatoid arthritis (R₁g₁ factors). R₁g₁ anti Gm factors are inhibited by large amounts of sera which are negative for the given Gm¹ character while S₁g₁ factors are not. Furthermore S₁g₁ factors are inhibited by very small amounts of serum positive for a given factor.

The anti Gm factors are usually isospecific and do not react with γ globulin from other species (11).

In contrast the specificities of rheumatoid factors which do not detect genetic character are much discussed (see 26). Some authors

have claimed that the reactivity with γ G-globulin of different species is only due to cross-reacting factors (1, 4). However, accumulating evidence shows that rheumatoid factors with species-restricted specificity do exist as well as cross-reacting factors (14, 15, 26, 29).

In the present study, results of agglutination, absorption and inhibition experiments indicated that rheumatoid factors in sera from blood donors behaved in a similar way. Separate factors reacting with rabbit γ globulin only, with human γ -globulin only, or with both, were demonstrable by absorption experiments. In inhibition experiments even heat denatured γ G-globulin only partly inhibited the activity against rabbit γ -globulin (the Warler-Rose test) with some of the sera.

Heat denaturation mainly changes sites on the Fc fragment with which rheumatoid factors react, causing rupture of disulphide bonds and intermolecular bridging (7). Antigen-antibody reaction on the other hand, mainly causes changes of the Fab fragment of the molecule (8, 17). It is thus reasonable that heat denatured γ -globulin will be more prone to give cross-reactions than γ -globulin altered in an antigen-antibody complex like an agglutinate. Rheumatoid factors may, however, also react with heat denatured Fab fragments (9).

Various findings further indicate that the antibody is more extensively changed in precipitation reactions than in agglutination. Aho & Simons (1) observed that immune precipitates containing γ G-globulin as antibody were able to absorb all rheumatoid factors present in a given serum. Even precipitates with Gm(1) γ G-globulin absorbed an anti-Gm(1) factor. By this procedure rheumatoid factor was also absorbed using autologous γ G-globulin.

A corresponding finding was made with anti- γ -globulin factors detected by pepsin digested γ -globulin. Such factors were not inhibited by undigested native γ -globulin and were not absorbed by red cells sensitized by whole anti-D. In contrast, immune precipitates containing whole γ G antibodies removed the activity (6). Obviously then, the reactivity of various rheumatoid factors and other anti- γ -globulin factors depends on the state of the γ -globulin used for their detection.

Various recent findings indicate that autospesific rheumatoid factors, i.e. factors which react with the individual's own native γ G-globulin, exist besides the isospecific and heterospecific factors (3, 11, 20, 27). Such factors may complex with the autologous γ -globulin *in vivo* and cannot be demonstrated in whole serum (3). Anti- γ -globulin factors detected by pepsin-digested γ G-globulin (16, 19) and probably also anti-antibodies (see 19), show primary specificity for autologous γ G-globulin.

The present results clearly show that rheumatoid factors in normal human sera possess similar specificities as were earlier shown with rheumatoid arthritis sera (14, 15, 26, 29). These findings indicate that the rheumatoid factors express only a qualitative and not a quantitative deviation from the normal state (25).

Fourteen of the positive sera were selected for more extensive studies including the anti CD Rubelex and the latex tests.

Agglutination, absorption and inhibition experiments indicated that the reactions with human and rabbit γ globulin were produced by two and possibly three factors besides cross reacting factors. Accordingly, rheumatoid factors in normal human serum and in rheumatoid arthritis serum showed similarly restricted specificities. Only quantitative and not qualitative differences seemed to exist between rheumatoid factors in the two types of sera.

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CORRELATION OF DNA BASE COMPOSITION AND ACID FORMATION FROM GLUCOSE OF STAPHYLOCOCCI AND MICROCOCCI

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Classification of gram positive catalase positive cocci has been a point of controversy since the early days of bacteriology.

At times all these organisms were placed in only one genus—named either *Micrococcus* or *Staphylococcus*—at other times they were placed in two different genera *Micrococcus* and *Staphylococcus*.

The Subcommittee on Taxonomy of Staphylococci and Micrococci (1962a) has recognized two genera *Micrococcus* and *Staphylococcus* and proposed to separate staphylococci from micrococci by use of the ability of staphylococci to grow and produce acid from glucose when incubated under anaerobic conditions.

DNA base composition clearly divides gram positive catalase positive cocci into only two groups: one having a high percentage of guanine + cytosine (65–75 per cent) and one having a low percentage (30–36 per cent). Studies by Silvestri & Hill (1962) and by Boláček *et al.* (1962) indicate that strains with high per cent GC values are micrococci and strains with low per cent GC values are staphylococci. Silvestri & Hill identified their strains as micrococci and staphylococci on the basis of a taxometric analysis. Boláček and his colleagues distinguished the two kinds of strains on the basis of the ability to produce acid from glucose anaerobically.

The main purpose of the present study was an analysis of the correlation between DNA base composition and the ability to produce acid from glucose among gram positive catalase positive cocci. Thirty different strains on which DNA base composition had been made by the above mentioned authors were collected and subjected to a number of different tests including potentiation of pH determinations of aerobic and anaerobic cultures in a medium containing glucose.

MATERIAL AND METHODS

Origin of Strains and per cent GC Data

30 strains of gram positive and catalase positive cocci were received from the National Collection of Type Cultures (NCTC), London and from the Czechoslovak Collection of Microorganisms (CCM), Brno (Table 1)

TABLE 1
List of Strains Used

Name	Coll number	Other number or names
<i>Micrococcus luteus</i>	CCM 132	
	CCM 840	
	CCM 1674	
"	CCM 337	<i>Sarcina lutea</i> ATCC 382
	CCM 169	<i>M. lysodeikticus</i> ATCC 4698
"	CCM 855	<i>S. fermentans</i> NCTC 2665 NCIB 9278 Hill 46
<i>roseus</i>	CCM 679	ATCC 186 NCTC 7523 NCDO 975 Hill 41
	CCM 837	ATCC 416 NCTC 7518
"	CCM 385	ATCC 185 NCTC 7515 NCIB 9149
	CCM 560	ATCC 179 NCTC 7514
"	CCM 633	ATCC 412 NCTC 7517 NCIB 8175
<i>spp</i>	CCM 740	
	CCM 836	
"	CCM 2087	
"	CCM 825	
<i>Staphylococcus</i>		
<i>afementans</i>	NCTC 7563	Hill 48
<i>roseus</i>	NCTC 7511	ATCC 144 NCDO 974 Hill 38
"	NCTC 7512	ATCC 177 CCM 557 Hill 39
<i>aureus</i>	NCTC 4136	Hill 1
"	NCTC 4163	Hill 2
"	NCTC 6571	ATCC 9144 Oxford II NCDO 907 Hill 3
	NCTC 8532	ATCC 12600 NCDO 949 Hill 4
	CCM 529	
<i>epidermidis</i>	CCM 901	<i>Micrococcus cereolyticus</i> ATCC 12559
<i>saprophyticus</i>	NCTC 7292	NCIB 8711 NCDO 948 Hill 21
	NCTC 7612	NCDO 781 Hill 23
<i>faecalis</i>	NCTC 189	Hill 29
"	NCTC 7564	Hill 31
	NCTC 7944	Hill 32
	CCM 1400	

CCM	Czechoslovak Collection of Microorganisms
NCTC	National Collection of Type Cultures
ATCC	American Type Culture Collection
NCIB	National Collection of Industrial Bacteria
NCDO	National Collection of Dairy Organisms
Hill	Strain no. used by Hill (1959) Silvestri & Hill (1965) Hill et al (1965)

The data on per cent GC values of the NCTC strains were drawn from the results of Silvestri & Hill (1965). The per cent GC values of the CCM strains were communicated by Boháček et al (1966).

The strains were kept on nutrient agar plates (medium A) with the following composition: beef heart 500 g, peptone 10 g, NaCl 3 g, Na_2HPO_4 (12 H₂O) 2 g, tap water 1000 ml, agar 17.20 per cent. All test media were inoculated from overnight cultures grown at 35°C on this medium.

Morphology and motility were determined by micro-technique of an overnight plate culture incubated at 35°C suspended in tap water. Gram staining was performed by the following method: crystal violet, 1 per cent aqueous solution 1 minute, Lugol's iodine solution 1 minute, ethanol 96 per cent $\frac{1}{2}$ minute. Counterstaining with carbol fuchsin.

Acid Production from Carbohydrates

A) In fluid medium. Ability to produce acid from glucose aerobically and anaerobically in a fluid medium was tested electrometrically with pH meter 11M 22 k (Radiometer, Copenhagen). The following medium was used: trypticase (Difco) 10 per cent, yeast extract (Difco) 0.5 per cent, NaCl 0.5 per cent, KH_2PO_4 0.1 per cent, glucose 10 per cent, distilled water (Frans *et al.* 1955). Cultures were grown in cotton plugged 150 x 15 mm test tubes with 5 ml medium. Anaerobic conditions were obtained by use of an evacuated anaerobic jar (Balck & Tallack, London Ltd). The last trace of oxygen was removed by addition of an appropriate volume of hydrogen after evacuation. After inoculation at 35°C, readings were made on the second, fourth and the sixth day. Control tubes containing the same medium without glucose were inoculated and pH was measured after anaerobic incubation for 6 days. Uninoculated control media were also examined.

pH 7.2 (Subcommittee 1955b) for determination of anaerobic production of acid from glucose and mannitol the medium was dispensed in an 80 mm layer. After inoculation the medium was covered with a 25 mm layer of sterile paraffin oil. A change of colour from purple to yellow throughout the tube within 6 days was recorded positive. In the test for aerobic acid formation from glucose, mannitol, galactose, lactose, maltose and mannose the medium was dispensed in layers 25 mm high. The tubes were inoculated by stabbing to the bottom of the tube. Incubation was at 35°C, and readings were made daily for 6 days.

Other Tests

Arginine reaction. This test was carried out by two methods.

(I) As a growth experiment in the following medium: peptone (Orthana special) 0.5 per cent, beef extract (Lab. Lemco) 0.5 per cent, trypticase (Difco) 1.500, 0.5 per cent, cresol red (1:500), 0.25 per cent, 1 (+) arginine monohydrochloride 1.0 per cent, 1/15 M H_2PO_4 , 1.1 per cent, 1/15 M KH_2PO_4 , 0.9 per cent, distilled water. Adjusted to pH 7.2.

(II) As a growth experiment in the following medium: peptone (Orthana special) 0.5 per cent, cresol red (1:500), 0.25 per cent, 1 (+) arginine monohydrochloride 1.0 per cent, 1/15 M H_2PO_4 , 1.1 per cent, 1/15 M KH_2PO_4 , 0.9 per cent, distilled water. Adjusted to pH 7.2.

Control media of (I) and (II) were prepared with ut arginine. The media were dispensed in cotton plugged tubes 155 x 9 mm both with and without a cover of a 25 mm layer of sterile paraffin. Medium (I) was inoculated lightly with a straight inoculating needle. Medium (II) was inoculated by pipette with 0.2 ml of a suspension made from three loopfuls of a fresh strain agar culture in 1 ml distilled water. Incubated at 35°C with uninoculated controls. Readings after 2 and 4 hours and daily for 5 days. Development of a colour change was recorded as positive. All controls remained unchanged in colour.

Catalase. A small amount of plate material was deposited by means of a glass rod in a drop of 3 per cent H_2O_2 on a slide. The effervescence of gas bubbles was read as positive.

Coagulase. The production of free coagulase was tested by heavy inoculation of cotton plugged tubes 155 x 9 mm (agar 1.5%) with horse blood is drawn to a final volume of 200 ml in bottles containing 20 ml of a 10 per cent sodium citrate solution. Separation of plasma from red corpuscles is achieved by standing for 24 hours. Dispensed in tubes in 3 ml amounts and incubated at 35°C. Read after 2, 4 and 18 hours. Definite clot formation was recorded positive.

Deoxyribonuclease. A nutrient agar medium (medium A composition given above) with 0.2 per cent of the sodium salt of thymus gland deoxyribonucleic acid (BDH) was streak inoculated and incubated for 20 hours at 35°C. Plates were then flooded

with 1 N HCl and clearing of the medium around the areas of growth was recorded as positive (Jacobs *et al* 1964)

Gelatin hydrolysis and H-S formation Observed by stab inoculation of 15x X mm tubes sealed with paraffin treated corks with the following medium Liebig's meat extract 0.75 per cent peptone (Parke Davis), 2.5 per cent, NaCl 0.5 per cent gelatin 12 per cent, FeCl₃ (4 H₂O), 10 per cent Incubation at 22° C and inspected daily for 14 days

Haemolysis Haemolysis was observed on nutrient agar plates (medium A composition given above) containing 11 per cent citrated horse blood The appearance of a clear zone around single colonies after incubation at 35° C observed daily for 3 days was considered indication of haemolysis

Nitrate reduction A medium with 0.02 per cent nitrate free KNO₃ and 0.5 per cent peptone (Bacto) was inoculated and incubated at 35° C for 20 hours The presence of nitrite was detected by adding five drops of 0.8 per cent sulphamyl acid in 5 N acetic acid and five drops of 0.5 per cent a naphthylamine in 5 N acetic acid A red colour indicated the presence of nitrite The presence of unreduced nitrate was checked by the addition of a small amount of metallic zinc dust Occurrence of a red colour indicated that nitrate was still present

Oxidase Tetramethyl para phenylenediamine dihydrochloride (1 per cent) in distilled water was streaked on to filter paper Culture from a nutrient agar plate was smeared on the impregnated paper The development of a strong blue colour within 10 seconds was registered as positive (Kovacs 1956)

Phosphatase Production of phosphatase was tested in the following medium peptone (Orthana special) 1.25 per cent, beef extract (Lab Lemco) 0.75 per cent NaCl 0.5 per cent agar (Japan) 1.2 per cent, 10.0 per cent CaCl₂ (2H₂O) 0.02 per cent distilled water and the sodium salt of phenolphthalein diphosphate in a final concentration of 0.01 per cent (Barber & Kuper 1951) Streak inoculated plates were incubated at 35° C for 18 hours Free phenolphthalein was detected by placing a few drops of an ammonia solution (about 25 per cent NH₃) in the lid of each Petri dish The development of a bright pink colour was recorded as positive

Pigmentation Pigment formation was examined in plate cultures incubated for 20 hours at 35° C and then for 3 days at room temperature (20-22° C) The following medium was used peptone (Difco) 0.5 per cent yeast extract (Difco) 0.5 per cent glucose 1.0 per cent agar 2.0 per cent pH adjusted to 7.2

Splitting of Tween 80 The presence of a Tween 80 splitting enzyme was demonstrated by spot inoculation of peptone agar plates containing peptone (Difco) 1.0 per cent NaCl 0.5 per cent CaCl₂ (2H₂O) 0.01 per cent agar (Bacto) 1.5 per cent Tween 80 1.0 per cent Activity was indicated by the presence of an opacity around the culture after 18 hours incubation at 35° C and a further incubation at room temperature for 4 hours (Sierra 1957)

Urease A urea splitting enzyme was demonstrated by two methods (a) by inoculation of a medium of the following composition peptone (Bacto) 0.1 per cent NaCl 0.5 per cent K₂HPO₄ 0.2 per cent urea 1.0 per cent phenol red (1.50) 0.6 per cent Distilled water Incubation at 35° C Daily readings for 11 days A red colour indicated a positive reaction (b) By making a heavy suspension of culture in the following solution K₂HPO₄ 0.1 per cent K₂H₂P₂O₇ 0.1 per cent NaCl 0.5 per cent phenol red (1.50) 0.1 per cent urea 1.0 per cent Control solution without urea Readings after 20 minutes 1 hour and 24 hours A red colour indicated a positive reaction All controls remained unchanged in colour

Voges Proskauer reaction A medium containing peptone 0.5 per cent K₂HPO₄ 0.5 per cent glucose 0.5 per cent and distilled water was inoculated and incubated at 30° and 35° C A sufficient number of tubes were inoculated to permit testing for acetoin production after 2 and 11 days incubation Tubes were placed in a slanting position in the incubator The test for acetoin was made by adding to the culture 1 ml of a 6 per cent alcoholic solution of α naphthol and 0.4 ml of 40 per cent KOH A red colour developed within 1 hour indicated the presence of acetoin

RESULTS

All strains (Table 1) were found to be non motile, gram positive and catalase-positive spheres in pairs and clusters

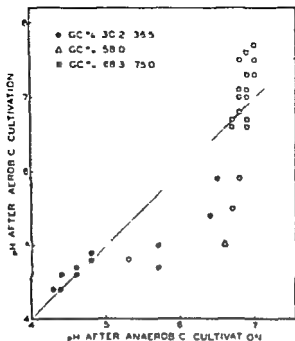


Fig. 1

pH values of cultures grown in a glucose medium for six days under anaerobic and aerobic conditions

Acid production from glucose under aerobic and anaerobic conditions as indicated by the pH of the cultures on the sixth day is shown in Fig. 1 and Table 2. The data of deoxyribonucleic acid (DNA) base composition are also given as reported by the examiners (Table 2).

In Fig. 1 the pH values of each strain are plotted in a diagram to show the relationship between the aerobic and the anaerobic pH values after cultivation for 6 days. By using separate symbols for strains with high and low per cent GC values the relationship between the pH values and the GC values is shown in the same diagram.

In Table 2 the same values are tabulated and here the strains are divided into three sections provisionally designated A, B and C by differences in their production of acid from glucose. Within each section the strains are arranged according to their anaerobic pH values starting with the strains showing the lowest values.

Section A contains 7 strains, all with low per cent GC values (30.2-36.5) and all showing pH values both aerobically and anaerobically. This level of acidification is reached already on the second day of cultivation. These strains were all received as *Staphylococcus* and 5 of them as *Staphylococcus aureus*.

Section C includes 15 strains, all with high per cent GC values (68.3-75.0 per cent) and all having pH values greater than 5, both aerobically

TABLE 2
pH Values of *Staphylococcus* and *Micrococcus* Cultures

Name and number of strains	Per cent GC values	pH values			Incubation six days		Section
		glucose medium		basal medium	anaerobic		
		anaerobic average (range)	No of readings				
A							
<i>Staphylococcus aureus</i> (CM 529)	34.2	4.3 (4.2-4.4)	2	4.4	7.2		
<i>Staphylococcus epidermidis</i> (CM 901)	35.6	4.3 (4.3-4.4)	2	4.4	7.2		
<i>Staphylococcus lactis</i> (CTC 7944)	36.5	4.4 (4.3-4.4)	2	4.6	7.1		
<i>Staphylococcus aureus</i> (CTC 4136)	32.8	4.6 (4.6-4.6)	2	4.7	7.2		
<i>Staphylococcus aureus</i> (CTC 8592)	31.2	4.6 (4.5-4.7)	2	4.6	7.1		
<i>Staphylococcus aureus</i> (CTC 6371)	32.0	4.8 (4.7-4.9)	5	4.8	7.1		
<i>Staphylococcus aureus</i> (CTC 4163)	31.2	4.8 (4.7-4.8)	2	4.9	7.3		
B							
<i>Staphylococcus lactis</i> (CTC 7564)	39.0	5.3 (5.0-5.3)	5	4.8	7.4		
<i>Staphylococcus saprophyticus</i> (CTC 7292)	31.6	5.7 (5.5-5.9)	6	4.7	7.1		
<i>Staphylococcus saprophyticus</i> (CTC 7612)	30.8	5.7 (5.3-6.1)	7	5.0	7.1		
<i>Staphylococcus lactis</i> (CM 1400)	30.2	6.4 (6.4-6.5)	3	5.4	7.1		
<i>Staphylococcus lactis</i> (CTC 189)	32.8	6.5 (6.4-6.8)	3	5.9	7.2		
<i>Micrococcus</i> sp. (CM 740)	58.0	6.6 (6.5-6.7)	2	5.0	7.1		
<i>Micrococcus</i> sp. (CM 2047)	68.8	6.7 (6.6-6.9)	3	5.5	7.6		
<i>Micrococcus</i> sp. (CM 825)	69.5	6.8 (6.7-6.9)	5	5.9	7.7		
C							
<i>Micrococcus</i> sp. (CM 836)	68.3	6.7 (6.6-6.8)	3	6.6	7.1		
<i>Micrococcus roseus</i> (CM 837)	69.7	6.7 (6.7-6.7)	2	6.7	7.1		
<i>Micrococcus roseus</i> (CM 679)	69.0	6.8 (6.8-6.8)	2	6.8	7.0		
<i>Staphylococcus roseus</i> (CTC 7512)	72.8	6.8 (6.7-6.9)	2	7.0	7.1		
<i>Staphylococcus roseus</i> (CTC 7511)	75.0	6.8 (6.6-6.8)	5	7.1	7.2		
<i>Staphylococcus fermentans</i> (CTC 7563)	72.8	6.8 (6.6-7.0)	5	7.5	7.2		
<i>Micrococcus luteus</i> (CM 132)	71.0	6.9 (6.9-6.9)	2	6.6	7.1		
<i>Micrococcus roseus</i> (CM 560)	71.0	6.9 (6.9-6.9)	3	6.7	7.1		
<i>Micrococcus luteus</i> (CM 385)	71.0	6.9 (6.9-6.9)	2	7.0	7.1		
<i>Micrococcus roseus</i> (CM 633)	72.8	6.9 (6.9-6.9)	2	7.1	7.3		
<i>Micrococcus luteus</i> (CM 337)	72.3	6.9 (6.8-7.0)	2	7.3	7.0		
<i>Micrococcus luteus</i> (CM 1674)	71.5	6.9 (6.9-7.0)	5	7.6	7.3		
<i>Micrococcus luteus</i> (CM 455)	73.0	7.0 (6.9-7.0)	2	7.3	7.1		
<i>Micrococcus luteus</i> (CM 169)	71.0	7.0 (6.9-7.1)	4	7.5	7.1		
<i>Micrococcus luteus</i> (CM 840)	71.0	7.0 (6.9-7.0)	5	7.7	7.0		
Uninoculated control		6.8 (6.7-6.8)	2	6.8	7.0		

and anaerobically, on the second fourth and sixth day of cultivation 12 of these strains were received as *Micrococcus* and 3 as *Staphylococcus*

Section B consists of 8 strains and is not homogenous either in pH values or in per cent GC values. Four strains have low GC values and the 4 other strains have high values. The pH values are intermediate between the values of the two other sections. It was observed that the pH values fell gradually throughout the observation period. This is in contrast to the results with the strains in sections A and C where the pH values reached their final value after only 2 days incubation and did not change essentially on further incubation. Five of the strains of section B were received as *Staphylococcus* and 3 as *Micrococcus*.

The accuracy of the pH determination by our method has been tested by calculation of the variance

$$s^2 = \frac{1}{n} \sum \frac{(x - \bar{x})^2}{n-1}$$

within strains of the results from cultures grown anaerobically for 6 days. Results of sections A and C strains showed a total variance of 0.0075 ($f=40$). Section B strains on the other hand displayed considerable inhomogeneity of results. The variance of section B was 0.09 ($f=26$), mainly due to the results of strains NCTC 7561 (variance 0.30 $f=4$) NCTC 7612 (variance 0.12 $f=6$) and NCTC 189 (variance 0.05 $f=2$). The cause of the large variance values of section B is not clear.

The results of the other tests performed on the strains are shown in Tables 3 and 4. Table 3 shows the results of all strains with low per cent GC values and includes the whole of section A and 4 strains of section B. Table 4 shows the results of all high per cent GC strains and includes the whole of section C together with the 4 strains of section B having high GC values.

It can be seen that the reaction patterns of the strains in Table 3 divide the strains into four small groups numbered 1 to 4 in the table. This subdivision seems to correlate with the final pH value in an aerobic glucose culture. However the differences are in some instances very small.

All the strains in Table 3 have low GC per cent values. The ability to metabolize arginine is found in all strains with low GC per cent values and is the only character which is not found in any of the strains having a high GC value (Table 4).

Fermentative production of acid from glucose as determined by indicator changes is common to the strains of section A.

The 4 strains of section B in Table 3 either vary in this respect (NCTC 7292) or only produce colour change in the top of the medium and therefore cannot be considered as true fermenters of glucose.

The strains in Table 4 all have high GC values. There is no unique

TABLE
Biochemical Characteristics of

Strains	pH in fluid glucose medium		Per cent GC values	Acid in semi anaerobic	
	Inoculated days			glucose	mannitol
	anaer (ave)	aer			
<i>Staphylococcus aureus</i> NCTC 8532	4.6	4.6	31.3	+	+
<i>Staphylococcus aureus</i> NCTC 6571	4.8	4.8	32.0	+	+
<i>Staphylococcus aureus</i> NCTC 4136	4.6	4.7	32.8	+	+
<i>Staphylococcus aureus</i> NCTC 4163	4.8	4.9	31.3	+	+
<i>Staphylococcus aureus</i> CCM 599	4.7	4.4	34.2	+	+
<i>Staphylococcus lactis</i> NCTC 7944	4.4	4.6	36.5	+	+
<i>Staphylococcus epidermidis</i> CCM 901	4.4	4.4	35.6	+	—
<i>Staphylococcus saprophyticus</i> NCTC 7292	5.7	4.7	31.6	or +	or —
<i>Staphylococcus saprophyticus</i> NCTC 7612	5.7	5.0	30.8		or —
<i>Staphylococcus lactis</i> CCM 1400	6.4	5.4	30.2		
<i>Staphylococcus lactis</i> NCTC 189	6.5	5.9	32.8		—

+ = positive — = negative — = weak reaction at top of the medium × = positive
No H₂S formation was observed. All strains hydrolysed urea by the method

character common to all strains and the reaction patterns do not allow of a subdivision of the strains into minor uniform groups. The results in the indicator tubes with glucose show that the majority of the strains do not produce acid; if they do the reaction is of the oxidative type. However the first strain in the table (NCTC 7564) must be characterized as a varying fermenter of glucose.

DISCUSSION

The purpose of our experiments was to evaluate and contrast two criteria recently proposed for division of the *Staphylococcus Micrococcus* group of bacteria viz the microbial formation of acid from glucose and DNA base composition (per cent GC values).

The data on DNA base determination of the *Staphylococcus Micrococcus* group so far published (Silvestri & Hill 1965; Boháček *et al.* 1965 and others) appeared very valuable as a basis of separation by showing a clear cut bimodal distribution of the GC per cent values. These were found to be either low (30–36 per cent GC) or high (63–73 per cent GC). The significance of the odd DNA base ratio of strain CCM 740 (Boháček *et al.* 1966) requires further study.

Fiats *et al.* (1955) examined acid production from glucose by clec

TABLE
Biochemical Characteristics of

Strains		pH in fluid glucose medium Incub 6 days		Per cent GC values
		anaerobic (average)	aerobic	
<i>Staphylococcus lactis</i>	NCTC 7564	5.3	4.8	69.0
<i>Micrococcus</i> sp	CCM 740	6.6	5.0	58.0
<i>Micrococcus</i> sp	CCM 2037	6.7	5.1	68.8
<i>Micrococcus</i> sp	CCM 823	6.8	5.9	69.5
<i>Micrococcus</i> sp	CCM 836	6.7	6.6	68.3
<i>Micrococcus luteus</i>	CCM 132	6.9	6.6	71.0
<i>Micrococcus roseus</i>	CCM 837	6.7	6.7	69.7
<i>Micrococcus roseus</i>	CCM 560	6.9	6.7	71.0
<i>Micrococcus roseus</i>	CCM 679	6.8	6.8	69.0
<i>Staphylococcus roseus</i>	NCTC 7512	6.8	7.0	72.8
<i>Micrococcus roseus</i>	CCM 385	6.9	7.0	71.0
<i>Staphylococcus roseus</i>	NCTC 7511	6.8	7.1	75.0
<i>Micrococcus roseus</i>	CCM 633	6.9	7.1	72.8
<i>Micrococcus luteus</i>	CCM 337	6.9	7.3	72.3
<i>Micrococcus luteus</i>	CCM 855	7.0	7.3	73.0
<i>Staphylococcus fermentans</i>	NCTC 7563	6.8	7.5	72.8
<i>Micrococcus luteus</i>	CCM 169	7.0	7.5	71.0
<i>Micrococcus luteus</i>	CCM 1674	6.9	7.6	71.5
<i>Micrococcus luteus</i>	CCM 840	7.0	7.7	71.0

+ = positive, — = negative, = weak reaction on top of the medium. No strains pro or anaerobically. The following tests were negative: coagulase, phosphatase, acetin, H₂S, arginine test under aerobic conditions when done as growth test (method 1), otherwise all

Section A is characterized by strong and fast acid production under anaerobic conditions. The strains all have low GC per cent values.

Section C is composed of strains with high GC per cent values and no capacity, or only a slight one, for aerobic or anaerobic production of acid.

Section B strains form small amounts of acid from glucose under aerobic conditions, and anaerobic formation of acid is slow and weak. It includes strains with both low and high GC per cent values.

The different levels of acidification in a glucose medium probably reflect differences in the method of glucose metabolism, and these differences might be of greater classificatory value than determination of pH levels. However, a determination of these characteristics was outside the scope of our study.

The biochemical characteristics of the 11 strains with low per cent GC values (Table 3) suggest the presence of four groups, although the small number of strains in each group do not permit definite recommendations. The 7 strains of section A seem divided into two groups by

19 Strains with High per cent GC

Acid production in semi solid medium			Nitrate reduction	Gelatin hydrolysis	Urease	Oxidation	Haemolysis	Pigment	Section
anaerobic	aerobic	galactose							
glucose	glucose	galactose							
or +	+	+	+	—	+	—	—	yellow	B
	+	—	—	+	—	—	—	orange	
	+	—	+	—	+	—	—	yellow	
—	+	—	+	+	—	—	—	orange	C
—	—	—	+	—	—	—	—	yellow	
—	—	—	+	—	—	—	—	pink	
—	—	—	+	—	—	—	—	pink	
—	—	—	+	—	—	—	—	pink	
—	—	—	+	—	—	—	—	pink	
—	—	—	+	—	+	—	—	pink	
—	—	—	+	—	—	—	—	pink	
—	—	—	+	—	—	—	—	pink	
—	—	—	—	+	+	—	—	yellow	
—	—	—	—	—	+	+	—	yellow	
—	—	—	—	—	+	+	—	yellow	
—	—	—	—	+	+	—	+	yellow	
—	—	—	—	—	—	+	—	white	

duced acid from mannitol mannose, maltose and lactose in semi solid medium aerobically deoxyribonuclease and Tween 80 splitting Strain CCM 1674 was positive repeatedly in the strains were negative in this test in both ways Both urease tests gave similar results

the coagulase, phosphatase and acetoin tests and also by a difference in GC per cent value level within the low GC per cent group

This indicates that minor differences in GC values within the complex having low GC values may be a useful guide for the internal subdivision by biochemical characters

The 4 section B strains with low GC per cent values seem divisible by differences in aerobic acid production of some carbohydrates and by the nitrate and acetoin tests

The strains with high GC per cent values (Table 4) appear inhomogeneous by the tests employed in this study and no internal subdivision is suggested by the results

A positive arginine reaction was common to all low GC per cent strains, in contrast to the negative reaction with the high GC level strains This is the only character found fully correlating with the DNA base ratio

A division of the *Staphylococcus Micrococcus* group according to DNA base ratio seems in principle the most acceptable one, especially

in view of the fact that two widely separated levels appear to exist.

We find that the proposal of the Subcommittee (1965a) to separate staphylococci from micrococci on the basis of their fermentative glucose catabolism fails to fulfil the necessary condition for its application, i.e. the presence of a clear-cut bimodal distribution in acid production from glucose in these bacteria. However, the test proposed by this Subcommittee may in most cases provide a convenient method in routine work.

The taxonomic position of the slow and late acid producers from glucose (section B strains) would be clear by accepting DNA base determination results as a basis for identification of such strains, but the laborious procedure by present methods prevents its application in routine work. The arginine reaction could be a useful substitute if proven to be fully correlated with the DNA base ratio determination in a large material of strains.

SUMMARY AND CONCLUSION

Gram-positive, catalase-positive cocci differ quantitatively in their anaerobic production of acid from glucose when determined electrometrically.

The differences in production of acid under anaerobic conditions do not permit a clear-cut separation in two groups and thus cannot be correlated with the bimodal distribution of per cent GC values of the strains.

Differences in both speed and strength of production of acid during anaerobic and aerobic cultivation do suggest a division by pH values of the strains in this study in three sections: one section of strong and fast producers of acid from glucose, all with low per cent GC (staphylococci), one section with little, if any, formation of acid from glucose, all strains with high per cent GC values (micrococci), one intermediate section with strains of both high and low GC values which slowly produces minor amounts of acid from glucose under anaerobic or aerobic conditions.

Biochemical characters of the strains with low per cent GC values indicate a possible non-arbitrary subdivision by per cent GC values: anaerobic and aerobic formation of acid from glucose, possession of coagulase and phosphatase, capacity of gelatin hydrolysis and splitting of Tween 80.

High per cent GC strains appear inhomogeneous in biochemical characters.

Among a number of biochemical tests, a positive arginine test was the only biochemical test fully correlating with a DNA base ratio of 30.2-36.5 per cent guanine + cytosine.

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SEROLOGIC INVESTIGATIONS ON TEICHOIC ACIDS FROM THE WALLS OF *STAPHYLOCOCCUS EPIDERMIDIS* AND *MICROCOCCUS*

By

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Received 23 viii 66

The teichoic acid in the wall of *Staphylococcus aureus* is of the ribitol type with alpha- or beta-linked N-acetylglucosaminyl residues. It has been shown by agglutination inhibition (13) and by agar gel precipitation (6, 3) that the configuration of the glycosidic linkage determines the serologic specificity. Thus two antigenic activities are found in the wall teichoic acids of *Staph aureus*.

In *Staph epidermidis* the wall teichoic acid is of the glycerol type with alpha- or beta-glucosyl residues (2). The serologic specificity on agar gel precipitation of a preparation containing glucosyl glycerol teichoic acid isolated from a *Staph epidermidis* strain was demonstrated by Lonnegård & Oeding (11). This antigen was considered to be identical with carbohydrate B described by Julianelle & Wiegand (10). Morse (12) isolated a glycerol teichoic acid containing glucose from another strain of *Staph epidermidis* (Prengel). The preparation gave a precipitation line on agar gel against homologous antiserum and precipitin inhibition studies suggested that the antigenic determinant was alpha linked glucose. Preliminary serologic studies on some *Staph epidermidis* teichoic acid preparations have been reported in (2).

The *Micrococcus* group seems to comprise strains which have either ribitol, glycerol or no teichoic acid in their walls (1).

In the present paper the antigenic activity of wall teichoic acids from some strains of *Staph epidermidis* and micrococci have been studied.

MATERIAL AND METHODS

Teichoic acid was isolated from the walls of five *Staph epidermidis* strains and two micrococcal strains as previously described (1). The teichoic acids are shown in Table 1 (see also (2)). From two of the strains (T1, T2) also other extracts were prepared (see below). The following purified preparations were used as references in the serologic investigations: polysaccharide A (beta glucosaminyl ribitol teichoic acid (5) and polysaccharide 263 (alpha glucosaminyl ribitol teichoic acid

(9) isolated from *Staph. aureus* strains Wood 46 and 263 KSF C respectively by saccharite 12:4 (11) (glucose and glycerol teichoic acid) is isolated from *Staph. epidermidis* strain 1234 (11) and protein A is isolated from *Staph. aureus* strain Cowan 1 (4). These preparations produce specific precipitation lines in agar gel in high dilutions when diffused against their homologous antiserum. The bacteria used in the agar diffusion experiments were live 18 hr cultures on nutrient agar suspended in a small volume of saline. Bacterial extracts were prepared in phosphate buffer pH 7.4 24 hr at 37°C and the supernatants after centrifugation were used.

TABLE 1
Wall Teichoic Acids Examined Serologically

Strain	Phenol	Hydrolysis in alkali*	Sugar*
<i>Staph. epidermidis</i>			
T1	Glycerol	Polylglycerol phosphate stable to alkali	Alpha glucose
T2	"	Polylglycerol phosphate stable to alkali	Beta glucose
12	"	Diglycerol monophosphate and beta glucosyl glycerol	"
7291	"	Diglycerol monophosphate and beta glucosyl glycerol	"
11242 (hyleus)	"	Glycerol diphosphate and saccharinic acid	Alpha glucosamine
<i>Micrococcus</i>			
13	"	Glycerol diphosphate and saccharinic acid	"
234	Bile salts	Bile salts phosphate stable to alkali	Glucose and glucosamine

* See (2).

Antisera were prepared by injections of formalin killed bacteria in rabbits (14). Ring test precipitation and precipitation by agglutination were performed as earlier described (7). Agar gel precipitation in Petri dishes was performed essentially as described in (8). However when the wells were filled simultaneously with anti-serum and teichoic acid in varying concentrations, no precipitation lines were observed. In a series of experiments the time of application of antiserum and teichoic acid as well as the inoculation temperature were then varied. The lack of precipitation lines using the standard technique was eventually found to be due to the unusually rapid migration in the agar gel of the teichoic acids. When the serum well was filled and then inoculated at 3-4°C for 1 hr, the filling of the antigen wells distinct precipitation lines were present after 4-48 hr at 4°C even in high dilutions of the preparations.

Standard solutions of the teichoic acids and saccharides containing 1 mg dried material per ml were diluted in saline. The antigen in dilutions of the standards

RESULTS

Staph. epidermidis T1. The wall of this strain contains glycerol teichoic acid with alpha linked glucose. Four preparations were examined: one was produced by the standard technique (1), the second by a single trichloroacetic acid extraction for 46 hr at 4°C, the third by saline extraction for 18 hr at 4°C and the fourth by saline extraction first for 18 hr at 4°C and then for 24 hr at 37°C. The saline extracts contained a trace of mucopolysaccharide in addition to glycerol teichoic acid.

TABLE 2
Ring Test Precipitation

Teichoic acid	Serum					
	T1	T2	I2	11249	I3	Wood 46
<i>Staph. epidermidis</i>						
T1	1:400	-	-	-	-	-
T2	-	6:400	3:200	-	-	-
I2	-	6:400	6:400	-	-	-
7291	-	3:200	-	-	-	-
11249	-	10	-	12:800	-	1
<i>Micrococcus</i>						
I3	-	-	-	-	100	-
234	-	400	-	-	-	1:600

Conc. of teichoic acids 1 mg/ml
Reciprocal titre values

TABLE 3
Agar Precipitation Lines Produced by Teichoic Acids

Line teichoic acid T1	Line teichoic acid T2	Line teichoic acid I3	Line polys A
<i>Staph. epid.</i> T1	<i>Staph. epid.</i> T2	<i>Microc.</i> I3	<i>Microc.</i> 234
" 1254	" 12		
	" 7291		

Against antiserum T1 all four preparations produced a distinct precipitation line on agar diffusion in dilutions 1/1,600-1/3,200 and ring test precipitation in dilutions 1/3,200-1/6,400 (Tables 2 and 3). Identical results were obtained with antiserum 1254, and polysaccharide 1254 (B) gave a reaction of identity with the T1 line. None of the other teichoic acids or polysaccharides produced this line or gave a positive ring test precipitation in T1 antiserum.

T1 bacteria produced two distinct lines on agar gel diffusion against antiserum T1: one strong line on the antigen well side and a weaker one on the serum side. The latter was identical with the teichoic acid line. The strong line was produced also by 1254 bacteria but not by the other strains examined. The two saline extracts contained small amounts of the antigen corresponding to the strong line, whereas the other T1 or 1254 preparations did not produce this line. The antigen producing the strong line resisted trypsin treatment.

Cross-absorptions of T1 and 1254 antisera with the corresponding preparations resulted in negative ring tests and disappearance of the agar precipitation line, thus verifying the identity of their teichoic acid antigens. The absorptions did not remove antibodies against the strong line.

Staph. epidermidis T2 The wall of this strain contains glycerol teichoic acid with beta linked glucose in the form of disaccharide residues (2). Three preparations were examined two of which were extracted with trichloroacetic acid like the T1 preparations. The third preparation was obtained by saline extraction for 24 hr at 37° C.

Against T2 antiserum the T2 teichoic acid preparations gave a single precipitation line on agar diffusion in dilutions 1:1600, 1:3200 and a ring test titre of 1:6400. The serologic activity of the 46 hr trichloroacetic acid extract was weaker in both tests. The T2 teichoic acid line is identical with the precipitation line produced by teichoic acids from *Staph. epidermidis* 12 and 7291, and the teichoic acid from *Micrococcus* 231 gave ring test precipitation with T2 antiserum (see below). There was no cross reaction with T1 teichoic acid.

T2 bacteria gave two distinct precipitation lines on agar diffusion with homologous serum: one strong line on the antigen well side and a weaker one on the serum side. The latter was identified with the teichoic acid line. The strong line was not produced by the T2 teichoic acid preparations or by the bacteria of the other strains examined. The antigen producing the strong line was sensitive to trypsin.

Staph. epidermidis 12 The wall of this strain contains glycerol teichoic acid with beta linked glucose (2). A single precipitation line was produced with 12 antiserum in 1:1600 dilution of the preparation where the ring test titre was 1:6400. The line was identical with those produced by teichoic acids from *Staph. epidermidis* T2 and 7291. T2 and 12 teichoic acids cross reacted to the same titres on agar gel and ring test precipitation and cross absorbed the antisera. As 12 antiserum gave a prozone the T2 antiserum was preferred.

12 bacteria gave two precipitation lines on agar diffusion: one strong, often double line on the antigen well side and a weaker line on the serum side. The latter was identified with the 12 teichoic acid line. The strong line was not produced by the 12 teichoic acid preparation or by bacteria of the other strains examined. The antigen producing the strong line was resistant to trypsin.

Staph. epidermidis 7291 The wall of this strain contains glycerol teichoic acid with beta linked glucose in the form of monosaccharide residues. The preparation reacted with T2 antiserum which gave a reaction of identity with the T2 teichoic acid line and was positive in 1:3200 dilution in the ring test. Teichoic acid 7291 completely absorbed antibody response to the T2 teichoic acid line and the ring test precipitation.

Staph. epidermidis 11249 (*lyticus* 11) The wall of this strain contains an atypical glycerol teichoic acid. In contrast to teichoic acids from other strains of *Staph. epidermidis* (2) this polymer is a mixed diester with both phosphate and glucosamine phosphate (Table 1). The 11249 preparation reacted in high dilution with 11249 antiserum by ring test precipitation where no precipitation line was

observed on agar diffusion. Very weak ring test reactions were observed with antisera T2, 1254, and Wood 46, whereas the ring test was negative with I3 antiserum.

Micrococcus 13. The wall of *Micrococcus* 13 contains a similar glucosamine glycerol teichoic acid as *Staph. epidermidis* 11249 (1). The serologic activity of the I3 teichoic acid/I3 antiserum system was rather weak. Ring test and agar gel precipitation were positive only in 1/100 dilution of the preparation. A specific precipitation line not present in any of the other preparations was produced.

I3 bacteria gave two precipitation lines on agar diffusion: one strong line on the antigen well side and a weaker one on the serum side. The latter was identical with the I3 teichoic acid line. The strong line was not produced by the I3 teichoic acid preparation or by bacteria of the other strains examined. The antigen producing the strong line was sensitive to trypsin.

Micrococcus 234. The wall of this strain contains a ribitol teichoic acid with glucosyl and glucosaminyl residues. The 234 teichoic acid gave a positive ring test precipitation in 1/1 600 dilution with Wood 46 antiserum. The polysaccharide A titre was 1/2 000. The 234 teichoic acid produced a polysaccharide A line on agar gel precipitation in 1/400 dilution. The preparation also gave a positive ring test in 1/400 dilution with antiserum T2, whereas with this serum no precipitation line was observed on agar gel. Absorption of antiserum T2 with polysaccharide A did not significantly reduce the ring test titre against teichoic acid 234.

DISCUSSION

Four of the teichoic acids here examined are of the glycerol type with glucosyl residues. In one (T1) glucose is alpha linked; in the other three (T2, 12 7291) it is beta linked. The results indicate that in these *Staph. epidermidis* teichoic acids the serologic specificity is determined by the glycosidic linkage. There was no cross precipitation between the teichoic acids with alpha- and beta-linked glucose in the ring test and each of them produced a specific line on agar gel precipitation. The serologically specific polysaccharide 1254 was described by Jønsen & Oeding (11) as a glucosyl glycerol teichoic acid probably identical with Julianelle & Wiegand's (10) group carbohydrate (B) present in apathogenic staphylococci. Polysaccharide 1254 was shown to react identically with teichoic acid T1 serologically, and the dextrorotation of Julianelle & Wiegand's preparation indicates that also in their preparation glucose was alpha linked. The *Staph. epidermidis* teichoic acid described by Morse (12) was also shown to have alpha linked glucose as the serologically determinant group.

The teichoic acids from T2, 12, and 7291, all with beta linked glucose, cross reacted completely in the ring test and agar diffusion test. In the T2 teichoic acid glucose is in the form of divaldehyde residues, where as

in strain 7291 it is in the form of monosaccharide residues. This difference apparently had no influence on the serologic specificity.

It has earlier been demonstrated that in the ribitol teichoic acid characteristic of *Staph. aureus* glucosamine is either alpha- or beta-linked and each linkage is serologically specific (13-15). *Staph. aureus* strains appear as a rule to have both types of linkages, but certain strains have only one of these. The present investigation shows that also in the glycerol teichoic acids of *Staph. epidermidis* the sugar occurs in serologically specific alpha- and beta-linkages. The linkage may indicate two distinct types in *Staph. epidermidis* since both linkages do not appear to occur simultaneously in the same strain as demonstrated in *Staph. aureus*. It remains to be established, however, whether all strains of *Staph. epidermidis* have the same teichoic acid, i.e. glucosyl glycerol teichoic acid or whether other types of teichoic acids are found (see strain 11219).

Micrococcus 13 and *Staph. epidermidis* 11219 (lysens) have similar very unusual glucosamine glycerol teichoic acids. Serologically, however, they do not cross react. Glucosaminyl linkages may be the reason for the very weak precipitation of teichoic acid 11219 in T2 1264 and Wood 46 antiserum. The teichoic acids of strains 11219 and 13 in spite of their structural similarity, are completely different serologically; this must reflect a difference in their configuration resulting in different determinant groups.

The teichoic acid from the most *Micrococcus* strain 234 cross reacts strongly in serum Wood 46 and T2. The production of a polysaccharide A line with serum Wood 46 indicates that glucosamine in teichoic acid 234 is beta-linked to ribitol and responsible for the cross reaction. Although teichoic acid 234 did not seem to produce the T2 teichoic acid line on agar diffusion, the strong ring test precipitation in serum T2 indicates that teichoic acid 234 has a second serologic specificity, probably determined by beta-linked glucose. As polysaccharide A or 263 antibodies were not demonstrated in serum T2, glucosamine is apparently not responsible for the precipitation of teichoic acid 234 in T2 antiserum.

This *Micrococcus* teichoic acid thus appears to have two serologic specificities and is related to *Staph. aureus* as well as *Staph. epidermidis* teichoic acids. A similar distinct cell wall polysaccharide AC was described by J. Svedgard & O. Ljung (11). The strain from which the polysaccharide was isolated, the 1113 strain of *Staph. aureus*, but according to types of teichoic acid and other characteristics, further investigations.

The present investigation also revealed precipitinogens present in whole bacterial cell material with the teichoic acid antigens. The importance of these substances for taxonomy and serologic typing remains to be established. The demonstration of one specific precipitinogen

in the bacteria of each of the strains T2 and I2, which have a common teichoic acid antigen, indicates that the bacterial precipitinogens have a narrower specificity than the teichoic acids.

SUMMARY

In glycerol teichoic acids isolated from strains of *Staph. epidermidis* alpha-linked glucose had one serologic specificity, beta-linked glucose another. The teichoic acids reacted in high dilutions and there was no cross-precipitation between preparations containing different linkages. They produced specific precipitation lines on agar diffusion.

Two very similar glucosamine-glycerol teichoic acids, one from a *Staph. epidermidis* strain (hyicus) and the other from a *Micrococcus* strain were completely different serologically. A ribitol teichoic acid with glucose and glucosamine from a meat *Micrococcus* apparently had two serologic specificities, one determined by beta-glucosamine linkages, the other by beta-glucose linkages.

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THE SO CALLED TOXOPLASMA HOSTILE FACTOR AND ITS RELATION TO ANTIBODY

By

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The effect on Toxoplasma kondii of specific antibody as revealed by the dye test is elicited only in presence of the so-called activator factors of "normal" (non-immune) serum. In the absence of antibodies the activator factors show no antitoxoplasma activity. However, *Sabin & Feldman* (21) in their first paper describing the use of the dye test for the diagnosis of toxoplasmosis also reported the occurrence of a heat-labile antitoxoplasma factor in normal sera of different mammalians. This factor was considered different from specific antibody as the antitoxoplasma activity of heat-treated normal sera could not like that of heat-treated immune sera be restored by the addition of activator serum.

Jettmar (12-13) has described a heat-labile antitoxoplasma factor occurring in the sera of several species including man. This factor was called the toxoplasma hostile factor (THF). *Jettmar* in contrast to *Sabin and Feldman* tested the sera without the addition of activator factors. The heat-lability of the THF might therefore have been due to destruction of activator factors such as heat-labile complement components originally present in the sera and necessary for the action of THF. *Grönroos* (7) re-investigating the THF considered the antitoxoplasma activity of normal serum to be the results of the combined action of activator factors and thermolabile toxoplasma antibodies both present in the sera.

The nature of the thermolabile antitoxoplasma factors described by *Sabin and Feldman* has not been properly established. In a review by *Feldman* (4) it was pointed out that all the sera of the animal species studied except those of mouse and man kill toxoplasma parasites in the absence of specific antibody. *Jacobs* (11) considered it possible that the antitoxoplasma activity in normal sera could be ascribed to low levels of non-specific antibody perhaps identical with properdin.

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With respect to the information available about the heat-lability of so-called "natural" antibodies (15), reaginic antibodies (3, 23) and the relatively greater sensitivity of 19S antibodies to heat compared to 7S antibodies (20) it seemed worthwhile to reinvestigate the nature of the antitoxoplasma activity of sera from normal (non-immunized) animals

MATERIAL AND METHODS

Normal sera (Non-immune sera) Rabbit, guinea pig monkey (*Cynomolgus*) and calf sera were obtained from apparently healthy individuals. These sera were designated as normal and the factor of the sera which exerted antitoxoplasma activity, in the presence of activator factors was referred to as the toxoplasma hostile factor (THF). The dye test titres of the normal animal sera varied from 4 to 160. The highest titres were observed in the rabbit sera and the lowest in the guinea pig sera.

Activator sera Human sera were obtained from apparently healthy blood donors without any previous known experience of toxoplasmosis and were tested for anti-toxoplasma activity. Selected sera which were free of antitoxoplasma activity were used as activator sera.

Immune sera A rabbit which had no detectable serum antitoxoplasma activity in a serum dilution of 1:4 was inoculated with about 1000 living toxoplasma parasites in the marginal ear vein. Ten days after the inoculation the rabbit was bled and the serum was used as a reference immune serum. The serum had a dye test titre of 2000 and a titre of 128 in the complement fixation test.

A human immune serum was obtained from a 24 year old man who had been diseased with the lymphoglandular form of toxoplasmosis for at least 3 months. This serum had a dye test titre of 8000 and a titre of 256 in the complement fixation test.

Parasites Toxoplasma parasites of the RH strain were used. A toxoplasma rich peritoneal exudate was obtained by inoculating Swiss albino mice intraperitoneally with toxoplasma parasites and then after 3 days harvesting the exudate. The exudate was centrifuged at $65\times g$ to remove most of the cells and cellular debris. The supernatant was recentrifuged at $440\times g$ and the sedimented parasites were resuspended to a concentration of 5 million to 10 million parasites per ml. This suspension was used in the tests described below.

Zymosan A preparation type A (batch 134/84) from Koch-Light Laboratories Colnbrook, England was used. Absorptions of sera to get RP or R3 were accomplished using 1 ml of a 0.2 per cent suspension of boiled zymosan for each 1 ml of the serum to be absorbed.

Properdin The same preparation of properdin (RdT1) (kindly supplied by AB H&B, Stockholm, Sweden) as described in an earlier paper (25) was used. This preparation containing 16 000 units properdin per gram was devoid of demonstrable complement components as measured with the aid of conventional reactions.

Test on morphological alterations (MA) of toxoplasma parasites Antitoxoplasma activity in normal as well as in immune sera was measured by means of MA tests. The test was performed as previously described (25) by mixing one part of test serum dilution with two parts of activator serum and one part of parasite suspension. Determination of the percentage of parasites with MA was made after one hour's incubation at $37^{\circ}C$. The MA observed were identical with those described as being characteristic of a positive dye test (21). The latter term is not used in the following, however, since no dye was used in the tests.

The amount of antibody or THF giving MA in 50 per cent of the exposed toxoplasma parasites in the presence of activator serum is in the following referred to as one antibody unit and one THF unit respectively.

Heat treatment An ultrathermostat LA DA type WB 20 was used. For each test 0.5 ml of the serum dilution was incubated in a glass tube for 30 minutes. Dilutions of sera were performed either with Hanks balanced salt solution pH 7.0 or with activator serum. The incubation temperatures employed ranged from 45° to $75^{\circ}C$.

Reduction with 2-mercaptoethanol The method of Schreihöfer *et al.* (22) was principally followed. The serum or serum dilution was dialyzed for 3 hours at room

temperature against 0.2 M 2-mercaptoethanol in 0.15 M β -hydroxy phosphate buffer pH 7.35. To prevent reassociation of dissociated macroglobulin alkylations were performed by dialyzing the sera against 0.05 M sodium acetamide in the same buffer for 4 hours at room temperature. Finally the sera were dialyzed overnight in the same buffer against the phosphate buffer for 7 hours at room temperature and then overnight in the same buffer.

Densitometric ultracentrifugation. The separation of antitoxins was performed in a Spinco model L ultracentrifuge using a preformed sucrose gradient ranging from 10 to 40 per cent. The sucrose gradient was made as described by Briffen & Roberts (2). After a dilution of sucrose to a final concentration of 10 per cent the centrifugation was run for 18 hours at 35,000 rpm using a SW 39 rotor. Serial fractions usually 13 were collected through a hole in the bottom of the tube. The fractions were tested for antitoxin plasma activity at varying dilutions.

Sephylux 400 chromatography. Gel filtration on Sephadex 400 was performed according to Floren & Kufanler (6). A column of the gel 100 cm in length and 3 cm in diameter was used. In each experiment two to 10 ml of serum was applied to the column and elution was carried out in the same buffer pH 7.35. The flow rate was 3.5 ml per hour and the eluate was collected in 5 ml fractions. The protein concentration of the fractions was determined by their absorption at 280 m μ . Concentration of fractions by gel filtration was performed either by ultrafiltration or by dialysis against 30-50 per cent polyvinylpyrrolidone followed by dialysis against the buffer used for elution. Antitoxins eluted in the first protein peak eluted in the chromogram will be referred to as 19% antitoxins and those eluted in the second peak as 7% antitoxins.

Block electrophoresis. Electrophoresis of sera was performed in a Leick block as described by Möller-Jherber (17). A 0.1 molar barbitol buffer pH 9.6 was used and a potential of 5 V per cm was applied for 20-24 hours. After the run the block was cut into 1 cm wide pieces and each piece was mixed with 0.5 ml of the buffer used. After thorough shaking of the mixtures they were centrifuged and the supernatants tested for antitoxin plasma activity. The protein content of the eluates was after dilution with distilled water determined by absorption at 280 m μ . The presence of immunoglobulins in the eluates was determined by means of immunoelectrophoresis (26) with the aid of a goat antirabbit globulin serum obtained from Microbiological Associates, Bethesda, Md. USA.

RESULTS

Dependence of THF on Activator Factors

All the investigated undiluted normal sera had a demonstrable antitoxoplasma effect but this effect could be abolished by diluting the sera 1:2 or 1:4. The antitoxoplasma activity of the diluted sera could however be restored after addition of human activator serum.

Portions of an activator serum were treated to destroy selectively various complement components (14). The variously treated activator portions were thereafter mixed with normal rabbit serum diluted 1:2 and the mixtures were tested for their ability to cause MA of toxoplasma parasites. A completely abolished or a markedly reduced THF-activating capacity of the activator serum was encountered after treatment of the activator serum with heat (56-60 minutes) or NH_4OH or absorption with zymosan at 37°C (Table 1). Addition of EDTA to a final concentration of 0.02 M to a mixture of THF and untreated activator likewise resulted in a loss of the antitoxoplasma activity. A mixture of THF and zymosan absorbed (17°C and 37°C) activator regained partly its antitoxoplasma activity after addition of properdin at a final concentration of 10 units/ml.

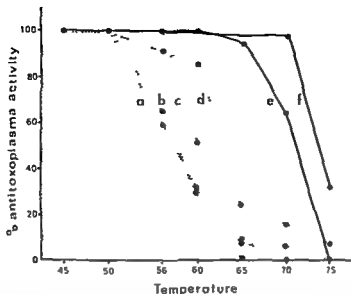


Fig. 1

The sensitivity to heat of TlIF compared to that of immune antibodies to toxoplasma

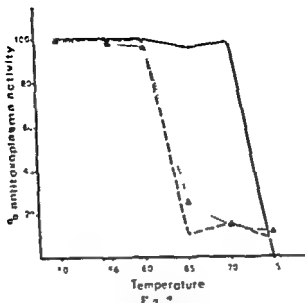
The results of heat treatment of the TlIF in normal calf (a) guinea pig (b) monkey (c) and rabbit serum (d) were compared with the results of treating immune antibodies in rabbit (e) and human (f) sera. For each serum solution containing four to eight antibody units were treated for 30 minutes at each temperature. Antitoxoplasma activity is expressed as percentage of the activity found in the untreated serum dilution. Mean values from tests on 3 normal sera from each species are plotted.

TABLE 1

The Effect of the Toxoplasma Hostile Factor in Presence or Absence of Activator Factors

Sample	Percent parasites with MA
TlIF	17
Activator	1
TlIF + activator	89
TlIF + activator heated at 56° C. for ½ hour	10
TlIF + activator absorbed with zymosan (37° C.)	51
TlIF + activator absorbed with zymosan (17° C. + 37° C.)	29
TlIF + activator treated with NH ₄ OH	27
TlIF + activator + EDTA	18
TlIF + progerdin + activator absorbed with zymosan (17° C. + 37° C.)	45
Progerdin + activator	6

The samples were mixed with toxoplasma parasites and after 1 hour's incubation at 37° C. the percentage of parasites with morphological alterations (MA) was determined for each sample. Absorptions with zymosan were performed for one hour at 17° C. and for half an hour at 37° C. Treatment with NH₄OH was performed according to the procedure described for obtaining H 4 (14). The final concentration of EDTA was 0.02 M and that of progerdin was 10 units per ml. TlIF stands for toxoplasma hostile factor, the source of which in this experiment was normal rabbit serum diluted 1:2.



The sensitivity to heat of THF compared to those of immune 7S and 19S antibodies in toxoplasma.

The results of heating normal rabbit serum (dotted line) and immune 19S (broken and 7S (solid line) antibodies are compared. Sera containing four antibody units were treated for each serum or serum fraction at each temperature for 30 minutes. The 7S and 19S antibodies were obtained in fractions of Sephadex G 200 chromatography of rabbit immune serum. Antitoxoplasma activity is expressed as percentage of the activity found in the untreated serum fraction or serum fraction.

Absorption of THF with *Toxoplasma* Parasites

Normal unheated fresh rabbit sera in varying dilutions were absorbed with a high concentration of toxoplasma parasites (1×10^8 parasites per ml) for 1 hour at 37° C. Absorption of sera containing up to two THF units with equal volumes of parasite suspension resulted in a total removal of the THF activity. The THF could be absorbed also when the absorption was performed at 4° C.

Influence of Heat on THF

Normal animal sera from each of eight guinea pig, monkey and rabbit origin were heat treated at the temperatures indicated in Fig. 1 for one-half hour. The sera had been diluted in Hanks' solution or human activator serum to contain 2–4 THF units. For comparison two immune sera containing both 7S and 19S antibodies as judged by Sephadex G 200 chromatography were also tested. The results of testing the anti-toxoplasma activity of the treated sera in the presence of activator serum are shown in Fig. 1.

It is evident from the figure that the THF in sera from the various animals showed different degrees of heat lability. Compared to the

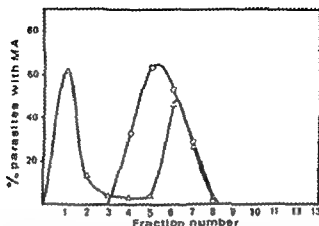


Fig. 3

Density gradient ultracentrifugation of THF and immune antibodies to toxoplasma: The results of ultracentrifugation in a sucrose gradient of one immune rabbit serum and one normal rabbit serum are illustrated. The antitoxoplasma activity is indicated as percentage of toxoplasma parasites showing MA after exposure to the serum fractions in the presence of activator. The immune serum tested was in other experiments by gel filtration (Fig. 4b) shown to contain 7S as well as 19S toxoplasma antibodies. Circles indicate antitoxoplasma activity in normal serum fractions and triangles indicate activity in immune serum fractions diluted 1:20 before being tested.

antibody activity in the immune sera tested, the THF activity in the normal sera was in all cases considerably more sensitive to heat.

In order to further characterize the heat-sensitivity of the THF in relation to those of 7S and 19S antibodies, Sephadex G 200 fractions of the immune rabbit serum containing these antibodies and a normal rabbit serum were heat treated. Before heating the fractions were diluted to contain equal amounts of antitoxoplasma activity. As shown by Fig. 2 the 19S antibodies were more sensitive to heat than the 7S antibodies. The THF activity of normal rabbit serum had a sensitivity to heat similar to that of the 19S immune antibody activity.

Reduction with 2-Mercaptoethanol

The THF in all the normal rabbit, guinea pig, calf and monkey sera studied was found to lose its activity after treatment with 2-mercaptoethanol. Experiments with 7S and 19S Sephadex fractions of the rabbit immune serum showed that the 19S antibody activity was completely abolished by treatment with 2-mercaptoethanol. At the same titre levels the 7S antibody titres were, however, also affected by this treatment and diminished about four- to eight fold.

Density Gradient Ultracentrifugation

Two normal rabbit, one guinea pig, one monkey and one calf serum as well as the immune sera of human and rabbit origin were fraction-

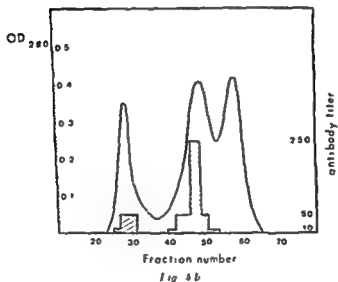
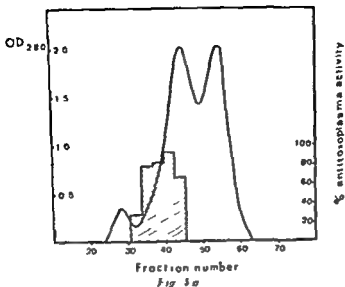


Fig 3

Gel filtration of THF and immune antibodies to toxoplasma

The results of chromatography on Sephadex G 200 column of normal rabbit serum (4 a) and rabbit immune serum to toxoplasma (4b) are illustrated. Solid curve represent optical density at 280 m μ . The antitoxoplasma activity measured as relative activity of undiluted fractions (Fig 4 a) or MIA test titre of the fractions (Fig 4b) is illustrated by hatched areas.

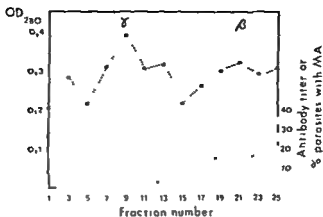


Fig 5

Pevikon block electrophoresis of THF and immune antibodies to toxoplasma

The electrophoretic mobilities of antibodies in rabbit immune serum and the THF in a normal rabbit serum are compared

The antitoxoplasma activity is expressed as MA test titre for the fractions of the immune serum or for the normal serum fractions, as percentage of toxoplasma parasites showing MA after exposure to the fractions in the presence of activator. Hatched area surrounded by dotted line indicates immune antibody activity and hatched area surrounded by solid line indicates THF activity. Dotted curve represents optical density at 280 mμ of diluted fractions.

ated by ultracentrifugation in a sucrose gradient. The THF or antibody activities of the fractions obtained were determined. The THF in all sera tested had a sedimentation rate which was equal to or slightly faster than that of the 7S antibodies. Fig 3 shows the results of an experiment where the sedimentation of the THF in normal rabbit serum is compared with the sedimentation of the 7S and 19S antibodies of immune rabbit serum.

Sephadex G 200 Chromatography

Normal sera from 5 rabbits, one monkey, one calf and 2 guinea pigs were fractionated by gel filtration on a Sephadex G 200 column. The antitoxoplasma activity was in all cases, except for one rabbit serum, which showed activity in both the first and second peak, located in fractions eluted slightly ahead of the second peak and extending into this peak. The maximum activity was for all the four rabbit sera which had only one peak of activity, eluted ahead of the second peak. Representative results are shown in Fig 4 which illustrates the results of gel filtration of one normal and one immune rabbit serum.

Block Electrophoresis

Normal sera from 2 rabbits, one monkey, one calf and one guinea pig as well as rabbit immune serum containing both 7S antibodies and 19S antibodies, were subjected to Pevikon block electrophoresis. By this technique the THF in the normal rabbit sera studied was found to have

a mobility similar to β -globulin (see Fig. 5). The zone of maximum activity was distinctly different from the zone of maximum activity of the immune serum tested. The mobilities of the THF in the monkey, calf and guinea pig sera were similar to γ -globulins.

DISCUSSION

The present investigation has given results indicating that what in this study has been called the toxoplasma hostile factor (THF) is a serum globulin which can be adsorbed to toxoplasma parasites and is dependent on complement factors for its action. The factor seemed in almost all the sera investigated to have characteristics which were different from those of IgM (19S antibodies). The results concerning the THF in rabbit sera are consistent with the hypothesis that the THF may be a fast moving immunoglobulin possibly of the IgA type. No conclusions regarding the frequency of the occurrence of THF being this type of immunoglobulin in various animals can be drawn. It should be noted however that of all normal animals tested only one had serum THF activity in IgM fractions. Since the factor occurs in normal serum it may tentatively be discussed as "natural antibody" although it cannot be excluded that the animals studied had previously suffered from toxoplasma infection. Natural antibodies have in recent studies (16-24) been shown to be immunoglobulins mainly of the IgM variety. Rabbit antibodies of the IgA type have been demonstrated by others. Onoue *et al.* (18) found that rabbit antioxyphen antibody could be IgA with a sedimentation coefficient of either 7S or 9S. Zwaifler & Beeler (25) reported results suggesting that rabbit antibodies capable of sensitizing rabbit skin for passive cutaneous anaphylaxis may be IgA.

Natural antibodies have in earlier studies been reported to be heat labile. Michael *et al.* (15) stated that "for each species immune antibody is more heat stable than natural antibody". They found a considerable variation between different species regarding the heat stability of natural antibodies. These results fit well with those of the present investigation where the toxoplasma hostile factors from different species showed different degrees of heat lability but were always more heat labile than the immune 7S antibodies tested. The differences noted could not be ascribed to higher concentrations of antibody in the immune sera than in normal sera since all sera were tested in dilutions containing approximately the same number of antibody units.

The heat lability of the natural antibodies against toxoplasma should be discussed in relation to the effect of heat on other antibodies studied. Pile & Schultze (20) found that 19S antibodies against various antigens were more heat labile than 7S antibodies against the same antigens. Svehag (24) reported that natural antibodies against poliovirus in rabbit sera were 19S globulins and were more heat labile than immune antibodies. Bermanic antibodies are markedly heat labile and are usually

sensitive to heating at 56° C for 30 min. (3, 23) There is suggestive evidence at hand that human reaginic antibodies usually are IgA (5, 8), although the recent findings reported by *Ishizaka & Ishizaka* (10) indicate that they may be immunoglobulins of another type.

The apparent sensitivity of toxoplasma natural antibodies to treatment with 2-mercaptoethanol might indicate that they are IgM or IgA. However, as *Wiederman et al* (27) have pointed out, the results of this sort of treatment do not offer any conclusive evidence regarding the nature of complement-fixing antibodies as it may very well occur that the complement-fixing capacity of IgG antibodies is destroyed by 2-mercaptoethanol without any effect on their combining efficiency. It was found in the present study that in most cases there was an evident lowering of the 7S antibody titres after treatment with 2-mercaptoethanol.

The THF in rabbit sera seemed to be dependent upon complement for its action although there was experimental evidence that it was neither IgG nor IgM. This finding is important in relation to the fact that in previous investigations (1, 9) IgA antibodies have not been found to fix complement. The rabbit skin-sensitizing antibodies described by *Zvaifler & Becker* (28) did not fix complement.

Concerning the origin of the THF nothing is known. As regards natural antibodies to viral antigens *Suchag* (24) has proposed that these antibodies are "associated with an immunological response to repeated stimulation with minute amounts of cross-reacting or identical antigenic determinants". The THF may possibly have a similar origin. The present results raise the question whether low serum dye test titres represent results of toxoplasma infection or merely stimulation by toxoplasma antigens present in foods. Low serum dye test titres are undoubtedly in many cases the result of a toxoplasma infection. However, the conclusions of any serologic survey using the dye test, with the aim to determine the prevalence of toxoplasma infection should be restricted to the findings of those higher titres commonly associated with generalized toxoplasma infection.

Properdin has by several authors been considered as being identical with natural antibody. In the present investigation properdin mixed with activator serum had no morphologically demonstrable significant effect on toxoplasma parasites in contrast to THF and activator. A properdin preparation previously (25) found to be devoid of demonstrable complement components could partly restore the antitoxoplasma effect of a mixture of natural antibodies and a zymosan-absorbed activator serum. Thus it seems clear that properdin and natural antibodies to toxoplasma are different entities. The claim that properdin is a distinct serum entity is in accord with the results of *Pensky et al* (19) who found that properdin does not share antigenic determinants with the known immunoglobulins.

SUMMARY

The so-called toxoplasma hostile factor was investigated in rabbit guinea pig calf and monkey sera. The factor was found to be antibody to toxoplasma showing different degrees of heat lability in various sera. By means of gel filtration, block electrophoresis, density gradient centrifugation and reduction with 2 mercaptoethanol the factor was in almost all cases shown to have characteristics different from those of IgM antibodies. The results suggested that the toxoplasma hostile factor in rabbit sera may be antibody of the IgA type. The dependence on complement and properdin for the action of the factor was demonstrated. The results indicate that the toxoplasma hostile factor and properdin are different entities. The origin of the toxoplasma hostile factor is discussed.

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BRIEF REPORTS

COUNTING THE NUMBER OF AEROBIC BACTERIA IN FULL-THICKNESS
HUMAN EPIDERMIS SEPARATED BY SECTION

By A. A. Mustakall and O. I. Salo, Rajjo Kistala and E. Kistala

Both direct and indirect sampling methods have been used for the study of the number of skin bacteria. The impression technique illustrates the density of the bacteria on the skin surface but does not differentiate between single bacteria and bacterial aggregates. The scrubbing, scraping and tape-stripping methods, while giving a measure of the deeper flora, suffer from the same drawback. In the scrubbing technique the application of a sampling fluid on the skin surface causes a more dispersion of bacterial aggregates but on account of individual variations in the penetration of the fluid it introduces considerable factors.

TABLE

Units of Aerobic Bacteria in Full Thickness Epidermis

Number of case	Sex and age	Diagnosis	Site of section	Area of biopsy in cm	Bacteria per cm
1	♀ 30	Healthy	Mids. lat. forearm	0.18	216
2	♂ 21	Healthy	Mids. lat. forearm	0.25	910
3	♂ 27	Healthy	Mids. lat. forearm	0.26	880
4	♂ 33	Healthy	Mids. lat. forearm	0.11	1370
5	♂ 33	Healthy	Mids. lat. forearm	0.41	146
6	♂ 25	Healthy	Mids. lat. forearm	0.59	119
7	♂ 32	Healthy	Mids. lat. forearm	0.1	61
8	♀ 18	Healthy	Mids. lat. forearm	0.10	5
9	♂ 33	Healthy	Mids. lat. forearm	0.12	194
10	♂ 27	Healthy	Mids. lat. forearm	0.47	83
11	♂ 15	Healthy	Mids. lat. forearm	0.31	37
12	♀ 19	Still	Upper arm	0.15	67
13	♀ 26	Eczema	Upper arm	0.65	3100
14	♂ 39	Eczema	Mids. lat. forearm left	0.12	860
			Mids. lat. right	1.00	700
15	♀ 34	Atopic dermatitis	Mids. lat. left site a	0.80	3100†
			Mids. lat. left site b	0.80	3100†
			Upper arm right	0.25	2900†
16	♂ 17	Atopic dermatitis	Upper arm right	0.01	250*
17	♂ 18	Atopic dermatitis	Mids. lat. forearm	0.01	3200†
18	♀ 16	Atopic dermatitis	Mids. lat. forearm	0.07	10000*

— Skin of normal appearance

† — Lichenified skin

‡ — *Staphylococcus aureus* present* — Culture of *Staphylococcus aureus*

kept in a non aggregated but viable condition

This note describes a suction biopsy technique fulfilling these requirements. Suction measuring 150-200 mmHg below atmospheric pressure results within three hours in a clean dermo-epidermal separation (1). The roof of the suction blister consists of a non-contaminated full thickness sheet of epidermis. The blister base reveals the original area of the epidermal sample. To recover the bacteria in a dispersed state the sample is gently homogenized in a glass homogenizer with a known amount of 0.075 M phosphate buffer (pH 7.9) containing 0.1 per cent of a non-ionic detergent Triton X 100. This solution has been successfully used for the dispersion of bacterial aggregates in a recent modification of the fluid scrubbing method (2). According to our experience it does not either alter the viability of aerobic bacteria within 24 hours. In the presence of the detergent the recovery of the bacteria was found to be about threefold as compared with counts obtained with buffer solution only. Homogenization was found to have no effect on the recovery of bacteria from the detergent solution.

The variation of the numbers of aerobic bacteria in the full thickness epidermal biopsies is illustrated in the table.

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IN VIVO INHIBITION OF THE GROWTH OF TRIC AGENTS BY PURIFIED SPECIES SPECIFIC INTERFERON

By V. Reinicke, C. H. Mordhorst and F. Schonne

TRIC (Trachoma Inclusion Conjunctivitis) agents constitute a group of organisms which are not true viruses but agents which have certain developmental and biochemical criteria in common with rickettsia and bacteria.

Previous experiments in this laboratory failed to show sensitivity of TRIC agents grown in ovo to relatively small virus inhibiting doses of crude chick interferon (2). Recently however sensitivity to the effect of interferon was demonstrated for a tissue culture (L-cell) grown TRIC-agent (1b1) in experiments employing high titered mouse interferon (3). Reinvestigation of the problem of in ovo sensitivity of TRIC agents has been performed in this laboratory using purified concentrated chick interferon (3). Crude interferon was prepared in chick embryos inoculated with influenza B Lee virus and purification was carried out by a six stage procedure consisting of 1. precipitation of virus and inactive proteins with 0.15 M HClO_4 , 2. precipitation of interferon with Zn and 3. resolution in HCl followed by dialysis to remove the Zn. The Zn precipitation 4. and resolution (5) were carried out twice and the material from the second precipitation was finally lyophilized (6) and used as purified interferon. The titres (50 per cent plaque inhibition end points) of crude interferon and purified interferon were 1:200 (200 units of interferon per ml) and 1:63,000 (63,000 units of interferon per ml) respectively.

The first experiments were concerned with the influence of interferon on the titres (FLD₅₀ = Egg Lethal Dose₅₀) of a stock suspension of the trachoma strain

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FOI II. Repeated experiments with varying doses of the FOI II strain showed that 1200 units of interferon inoculated into the yolk sac of the chick embryo (either three hours before or simultaneously with the FOI II strain) resulted in a clear cut growth inhibition of the FOI II agent. A representative experiment showed a titre of $10^{5.5}$ FID₅₀ per ml in the control embryo in contrast to $10^{-2.0}$ FID₅₀ per ml in the interferon treated embryo. Similarly purified interferon (1200 units) also decreased the death rate of THIC agent SS 10N inoculated embryos as revealed by a delay of two days in 50 per cent embryo mortality (after infection with $10^{-2.5}$ FID₅₀) in the interferon treated group (Table 1). Experiments failed to show any direct inactivation of THIC agents by interferon. Interferon in amounts with a definite inhibitory effect on the growth of the FOI II strain in chick embryos did not influence the growth of FOI II in duck embryos. (6) Two important criteria for inhibitory effect attributable to interferon were consequently fulfilled in the experiments described: 1. Lack of direct inactivating properties and 2. species specificity of the action.

Preliminary studies of the amount of interferon necessary for in vivo inhibition of THIC agents were also performed. It was found that ~ 100 units of interferon per embryo resulted in growth inhibition while ~ 200 units had a detectable inhibitory effect. Earlier experiments have shown that detectable inhibition of in vivo growth of influenza virus was obtained with a minimum of 50-100 units of interferon per embryo (5). Thus THIC agents seem to be considerably less sensitive than influenza virus to the action of interferon.

With the accumulating evidence that THIC agents are not viruses but organisms resembling bacteria (4) it is interesting that interferon generally considered to be a virus inhibiting substance is able to inhibit the growth of these agents. It remains a question whether the present observation can be extended to other intracellularly grown organisms that differ from viruses.

TABLE 1

The Influence of Interferon on the Death Rate of THIC Agent SS 10N Inoculated Chick Embryos

Days after inoculation of SS 10N ($10^{-2.5}$ FID ₅₀ embryos)	Interferon treated	Control
3		
4		
5		1
6		2
7	1	4
8	3	5
9	2	1
10	5	
11		
12	2	

The dotted lines show the time interval of 1 per cent embryo mortality

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HAIR NEOGENESIS IN RAT AND RABBIT SKIN

By E. Stenback, T. Aunimäki, A. Dammert

Until recently, the consensus has been that the population of hair follicles in the adult animal is fixed, the number being determined during foetal life. However, new formation of hair follicles has been reported by some authors. Some of the most important experiments in this field are those of *Breedis* (1) and *Taylor* (3). *Taylor* studied the healing of the skin of rat following freezing while *Breedis'* studies were based on healing of full thickness wounds in rabbit skin.

The present authors have repeated these experiments in order to study hair neogenesis in different conditions. The skin of 20 rats was frozen with carbon dioxide snow following the method described by *Taylor* (3). A carbon dioxide slab diameter 1 cm. was pressed onto the back skin of the rats with constant gentle pressure for 1 minute. This caused necrosis of the skin and a wound formed. Gradually the edges contracted and after 10 weeks the wounds were almost completely healed. Histological sections of the wounds were taken weekly for 10 weeks. Neither macroscopical studies nor the histological sections showed any signs of hair formation in the granulation tissue. In contrast to the studies of *Mikhail* (2) and *Taylor* (3) no neogenesis of rat hair could be observed.

Using rabbits a series of experiments was performed by the technique of *Breedis* (1). A metal ring was placed in the wound made in the back skin of 10 rabbits. The wound was protected by a glass cover. The animals were studied for 6 months. During the first days the upper layer of the loose subcutaneous tissue became attached to the loose edges of the wound. In 7-10 days the membrane thickened and became opaque. In 10-14 days a brown scab covered the entire wound. The granulation tissue growing in from the edges reached the centre in about 3 weeks. Epithelialization was usually complete a week later and the scab disappeared. In most animals small hairs became visible after about 2 months. They were white and no pigment could be seen. After 6 months a smooth covering of white hair covered the entire surface of the wound.

The present investigations support the concept of *Breedis* (1) that scar epithelium is capable of redifferentiating into hair follicles and sebaceous glands and that granulation tissue is capable of redifferentiating into dermal papillae. These findings indicate that cutaneous cells are pluripotent and equipotential throughout life at least in rabbits.

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ROUS RAT SARCOMA STUDIED WITH FERRITIN CONJUGATED ANTIBODIES

By

JARS G. RAN LINDBERG and PETER BIRNREIFELD

Received 5 ix 66

Certain strains of Rous Sarcoma virus (RSV) are known to induce sarcomas in rats (for references see Ziller 1965). Transfer of Rous rat sarcoma cells to chickens in which a Rous chicken sarcoma at the site of injection indicating the presence of virus information in the rat sarcoma cells. It is, however, not known in what form the virus information persists in the rat sarcoma. So far infectious virus has not been isolated from the rat tumors. Nor have virus particles been demonstrated by the electronmicroscope technique in the rat sarcoma cells. An attempt to demonstrate the presence of the virus using the fluorescent antibody technique was made in a previous study (Lindberg 1964). Antibodies against Rous sarcoma virus were produced in chickens. These antibodies were tested on living rat sarcoma cells which showed a specific fluorescence in the periphery of the cells. It was suggested that the rat sarcoma cells contained an antigen common to both chicken and rat sarcoma cells.

The aim of the present study was to investigate the occurrence of RSV antigens in the Rous rat sarcoma cells using ferritin labeled antibodies. The rat sarcoma was induced in 1961 with RSV strain S II by Ahlström & Jonsson and has since then been carried in series in rats. Transfers of the rat sarcoma to chickens almost invariably induced a virus producing Rous chicken sarcoma.

MATERIAL AND METHODS

Virus. Rous virus (RSV) strain SR was obtained from homogenates of rapidly growing chicken sarcomas purified by a low speed ultracentrifugation according to Moloney (1960). The virus preparation was suspended in 0.01 M citrate buffer at pH 6.8 and stored at -70°C until tested. Its activity after storage was more than 10⁵ i.u./ml when tested according to Temin & Rubin (1958).

RR sarcoma. The Rous rat sarcoma has the character of a rapidly growing

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anaplastic round cell sarcoma which usually kills the rats in 10-14 days. Retransfer of living rat sarcoma cells from every 10th passage into chicken induces a Rous chicken sarcoma of the same histological appearance as the original chicken sarcoma. The investigation was carried out on rat sarcoma cells obtained from ascitic fluid. The solid rat tumor (passage Nos 124 and 125) was finely minced with scissors and suspended 1:5 in phosphate buffer saline. 0.1 ml of the suspension was injected intraperitoneally into rats, aged 3-4 days. Ascitic fluid containing numerous sarcoma cells, almost 100 per cent of which were viable, was obtained seven days later.

RSV antiserum. Antibodies against RSV were produced in chickens at least 10 weeks old immunized according to the scheme suggested by Fink & Rauscher (1961). After three booster injections given at 3 week intervals the chickens were bled and serum was tested for virus neutralizing capacity. Serum diluted 1:100 gave at least 90 per cent inactivation. The test was performed according to Rubin (1960).

Antiserum against chicken γ globulin. Antibodies were produced in rabbits. The chicken globulin was purified according to Strauss *et al.* (1960). Immunization was done in a conventional way and the animals were bled when signs of an anaphylactic reaction was noticed after a small intravenous booster injection. The specificity of the rabbit serum was tested by immuno electrophoresis and the double diffusion technique in agar gel according to Ouchterlony (1949). Only serum producing a single clear-cut precipitation line against chicken γ globulin was accepted for further use.

Conjugation of immunoglobulin and characterization of conjugates. The procedure used for the conjugation of antibody and ferritin and the purification of conjugates is described in detail elsewhere (Hibersfeld *et al.* 1966a, b). Briefly the globulin fraction used for conjugation was obtained by sodium sulphate precipitation of the immune serum (Strauss *et al.* 1960). Ferritin (Pentex Inc., Kankakee) and globulin were conjugated by means of the bifunctional reagent toluene-2,4-diisocyanate (TC) essentially according to the procedure described by Singer & Schick (1961). Unconjugated globulin was removed by ultracentrifugation of the crude conjugate at 110 000 \times for 1½ hours.

The conjugate was fractionated further by column electrophoresis.

Only fractions immunophoretically free from unconjugated ferritin and globulin respectively, were used for incubation experiments. Agar diffusion tests according to Ouchterlony (1949) against anti ferritin serum and anti rabbit γ globulin serum confirmed the purity of the conjugate and precipitation lines showing partial fusion were obtained.

Testing of sarcoma cells. The rat sarcoma cells were tested for cellular antigens using the fluorescent antibody technique as a model (Wöller 1961). After the rat sarcoma cell suspension had been thoroughly washed in BSS (balanced salt solution) about 2×10^6 cells were incubated with chicken anti-RSV serum for 20 minutes at 37°C. The suspension was then washed three times in BSS and then incubated with ferritin labelled antibodies for another 20 minutes at 37°C, after which it was rewashed and immediately fixed and processed for electron microscopy.

In control experiments the sarcoma cells were treated with saline or chicken anti rabbit albumin serum prior to the incubation with the ferritin conjugate. The specificity of the reaction was also tested by blocking experiments with unconjugated anti chicken γ globulin serum.

Electron microscopy. The cell suspension was fixed in glutaraldehyde (6.5 per cent in saline) (Sabatini *et al.* 1963) for about 3 hours at 41°C and then post fixed in buffered OsO_4 (3.33 per cent OsO_4) in a collidine pH adjusted to 7.2 with phosphate buffer for 60 minutes, dehydrated in graded ethanol and embedded in Epon 812 (Luft 1961). The specimens were sectioned on a LKB Ultratome and examined in a Zeiss EM 9 electron microscope.

RESULTS

The rat sarcoma suspensions, incubated first with anti-RSV-antibodies and then with ferritin-labelled anti-chicken- γ -globulin antibodies, contained cells with conglomerates of ferritin, which appeared to be irregularly attached to a filamentous, branched substance (see Figs 1-2).



Figs 1 to 4 illustrate details of Rous rat sarcoma cells which have been treated with chicken anti RSV serum washed and subsequently inoculated with purified ferritin conjugated anti-chicken antibodies

Fig 1

Rous rat sarcoma cells showing conglomerates of ferritin located in the outer third of the cytoplasm $\times 40,000$ Detail of same conglomerates $\times 35,000$

Practically all the conglomerates were situated in the periphery of the cell as a rule just inside the outer cell membrane and never in the central part of the cell. Sometimes the ferritin particles and conglomerates were localized inside vesicles in the cytoplasm and as a rule only in those which were located close to the plasma membrane. Sometimes the ferritin conglomerates adhered to the vesicle walls. No tagging of the plasma organelles or of structures resembling RSV particles was observed.

In some of the sarcoma cells rather large conglomerates of ferritin were found as seen in Fig 1. In these cells the cell matrix around the conglomerates seemed to be looser or less homogenous than in normal tumor cells (see Figs 1 and 2). Careful study of the cell membrane of these cells revealed small parts of the membrane which seemed to be

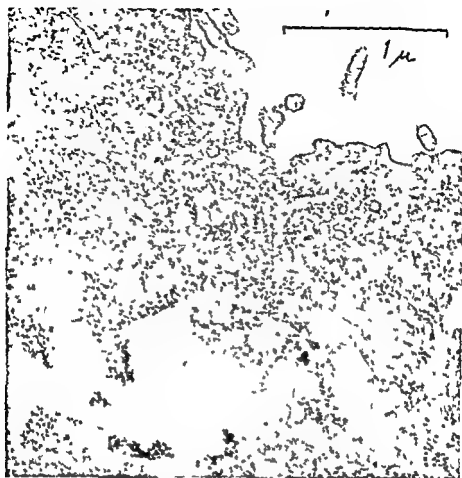
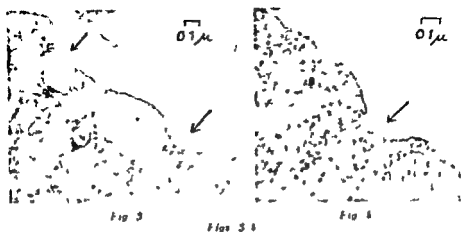


Fig. 2

($\times 40,000$) merates of ferritin particles arranged like filaments in the outer part of a Heus rat sarcoma cell. No conjugation to specific cell organelles. Note the swollen and loose appearance of the cell matrix around the ferritin conglomerates. $\times 40,000$

split or porous (see Figs 3 and 4). The appearance of the plasma membrane resembled that demonstrated by Jacoby & Wahren (1965) in experiments with Gross lymphoma cells treated with cytotoxic tumor specific antibodies.

In cells classified as dead the ferritin particles were scattered in the cytoplasm as well as in the nucleus. These cells usually showed vacuolization and disintegration of the cytoplasmic organelles, swollen mitochondria and parts of the plasma membrane disrupted or totally lacking. As a rule cells which were viable when fixed could readily be distinguished from dead cells. The random distribution of ferritin-conjugates in the cytoplasm of the dead cells is probably an unspecific diffusion phenomenon in analogy with the fluorescence of the nuclei and cytoplasm in dead cells in experiments using the fluorescent anti-



Part of Rous rat sarcoma cells tagged with ferritin and showing pericellular parts of the cell membrane $\times 45,000$ and $60,000$ respectively

body technique (Miller 1961). Apart from cells classified as dead, there were hardly any cells in the control preparations containing appreciable amounts of ferritin.

No ferritin was observed in sarcoma cells treated with saline. When unspecific immune serum (chicken anti rabbit albumin) was used instead of anti RSV serum, occasional ferritin particles were seen in the cytoplasm of the sarcoma cells. The percentage of tagged cells, however, was significantly lower and only exceptionally did they contain conglomerates comparable in size and appearance to those illustrated in Figs 1 and 2.

Sarcoma cells treated with chicken anti RSV serum and subsequently incubated with unconjugated anti-chicken γ globulin serum lost all their ability to bind ferritin conjugated anti-chicken globulin. Thus the ferritin-conjugated antibodies were prevented from reacting with the anti RSV antibodies in the sarcoma cells.

Unlike previous fluorescent studies (Hilgert 1961) it was not possible to determine the number of cells reacting with anti RSV serum. However, the number of cells with specific ferritin staining was roughly the same as that found to be positive in the fluorescence experiments, viz. about 20 per cent of the sarcoma cells reacted with anti RSV serum.

DISCUSSION

Earlier experiments using the fluorescent antibody technique have indicated that Rous chicken sarcoma cells and Rous rat sarcoma cells contain determinants with identical immune properties (Lindberg 1964).

In the present study the distribution of the RSV antigens in the rat sarcoma cells were investigated by means of the ferritin antibody

technique. In the rat sarcoma cell the ferritin particles were intracellularly localized to the peripheral zone of the cytoplasm and to cytoplasmic vesicles just below the cell membrane. This might indicate an antigen-antibody reaction in this region, which is in agreement with the previous immunofluorescent observations, where the fluorescence was found along the cell membrane. Some of the cytoplasmic vesicles, even those containing ferritin, might represent pinocytotic vesicles and it is possible that pinocytosis could in part explain the intracellular localization of the ferritin into the cell. However, mere pinocytosis cannot explain the fact that only cells treated with anti-RSV-serum contained vesicles with conglomerates of ferritin.

The intracellular localization of the ferritin conjugate implies injury to the cell membrane, presumably accompanied by altered osmoregulation (Goldberg & Green 1960). The RR-cell incubated with anti-RSV-serum showed slight membrane changes, resembling those described by Jacobsson & Wahren (1965). In addition the swollen appearance of the cytoplasm might indicate osmotic damage.

The results obtained so far do not allow any definite conclusions about the nature of the presumed antigen.

No Rous virus particles have been found, and no structures resembling virus particles have been seen in association with the ferritin conglomerates. It cannot, however, be excluded that the intracellular antigen is related to an incomplete Rous virus particle. The peripheral localization of the ferritin conglomerates indicates that the antigen is accumulated at the periphery of the cells. By histochemical staining it is possible to demonstrate in the Rous rat sarcoma cell a large amount of acid mucopolysaccharides, which has almost the same distribution as the ferritin conglomerates. Both the Rous chicken sarcoma and the Rous rat sarcoma are known to produce acid mucopolysaccharides, and an association between them and the antigen cannot be excluded.

The intracellular localization of the ferritin conglomerates must not be taken as definite evidence of an intracellularly located antigen. It is possible that the ferritin conglomerates owing to injury of the cell membrane have entered passively into the cell. The loose appearance of the cytoplasm is consistent with such an interpretation. It is evident, however, that a specific antigen-antibody reaction has occurred as no reactions at all could be observed in the control preparations.

Recent experiences using the transplantation technique indicate the presence of cellular antigens common to Rous mouse sarcoma and Rous rat sarcoma cells (Jonsson 1966) indicating that the sarcoma cell has antigenic determinants on the cell membrane, which can react with specific antibodies. The demonstration of specific complement-fixing antigens in hamster and guinea-pig tumors (Huebner *et al.* 1964) also indicates the presence of common antigens. However the specific anti-RSV-serum used in the present investigation was produced against purified homogenates of Rous chicken sarcoma tissue. Moreover, no

common transplantation antigens could be demonstrated between Rous chicken and Rous mouse sarcoma (Jonsson 1966). The precise nature and localization of the antigen (surface antigens?, incomplete virus?, antigens related to or connected with mucopolysaccharides?) is an open question.

SUMMARY

The occurrence and localization of antigens in Rous rat sarcoma cells reacting with anti RSV-antibodies were studied by the ferritin antibody technique. The antisera were obtained in chickens immunized with partly purified chicken sarcoma homogenate. The ferritin conjugated antibodies were found to react with some of the rat sarcoma cells. The reaction occurred at the periphery of the tumor cell but did not involve specific cell organelles or any structures resembling intact Rous virus particles.

The results indicate the presence of antigens common to Rous rat sarcoma and Rous chicken sarcoma.

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MALIGNANT CATECHOLAMINE PRODUCING TUMOUR OF THE CAROTID BODY

By

C. A. HAMBERGER, C. B. HAMBERGER, J. WERSALL and J. WÄLTERMARK

Received 2 ix 67

Tumours of the carotid body are usually benign slowly growing neoplasms (*Compere 1951*). Bilateral as well as multilocular forms have been reported (*Marcuse & Chamberlain 1956*, *Rush 1963*). Malignancy is rare and only a few cases have been reported in the literature (see review by *Pryse Davies et al 1964*).

Carotid body tumours producing catecholamines have been described earlier (*Bertal et al 1962*, *Glenner et al 1962*, *Pryse Davies et al 1964*). The present paper deals with such a malignant carotid body neoplasm which was studied with light microscopy and with the fluorescence method for the demonstration of certain monoamines (*Falck et al 1962*, *Falck 1962*, *Orrodi & Hillarp 1963*, *1964*).

CASE REPORT

The patient was a 60-year-old man suffering from hypertension (210/120). Among laboratory tests may be mentioned the urinary excretion of norepinephrine which was 211 µg per day (normal 33 ± 11 µg per day). He was operated upon for a carotid body tumour growing invasively around and in the adventitial layers of the right carotid artery. At the operation the carotid bifurcation had to be resected and replaced by a vein graft. The patient died four days afterwards from extensive infarction of the brain.

MATERIAL AND METHODS

Specimens were taken for light microscopy from the tumour during the operation and from the operation field and the lymph node metastases at the autopsy. These

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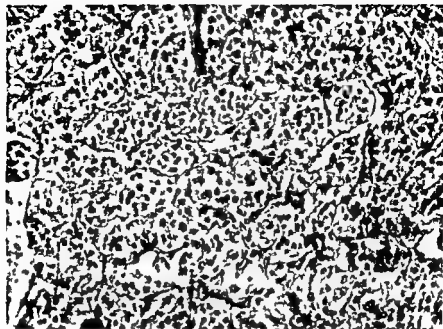


Fig. 1

Carotid body tumour composed of groups of round or polygonal epithelioid cells with a clear cytoplasm. Hematoxylin and Eosin. 200 X.

specimens were fixed in 10 per cent neutral formalin embedded in paraffin sectioned and stained with hematoxylin and eosin.

During the operation small parts of the carotid body tumour were also quickly frozen in propane cooled with liquid nitrogen freeze dried treated with formaldehyde gas at 80° for one hour embedded in paraffin wax sectioned and mounted for fluorescence microscopy. (For further technical details see Norberg & Hamberger 1964; Falek & Ouman 1965).

Urinary catecholamine excretion was determined according to Fuler & Ishajko (1961).

RESULTS

15

The carotid body tumour was composed of groups of round or polygonal epithelioid cells with a clear cytoplasm (Fig. 1). No mitoses were found. The scanty stroma was highly vascularized. Autopsy specimens from the operation field contained small islands of tumour cells infiltrating the connective and fatty tissue around the vessel transplant. The lymph node metastases consisted of tumour tissue with the same structure as that of the primary tumour.

After formaldehyde gas treatment strong fluorescence could be detected in the epithelioid cells of the carotid body tumour (Fig. 2). The intense fluorescence had the yellow green colour typical for certain catecholamines i.e. noradrenaline, dopamine and adrenaline. Only the cytoplasm of the cells showed specific fluorescence where as the nucleus appeared dark. No yellow fluorescence indicating the presence of 5-hydroxytryptamine was observed.

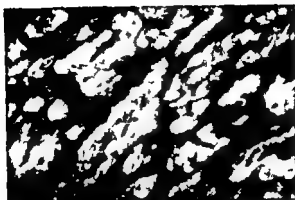


Fig. 4

Fluorescence micrograph of ear tilt adenoma of tumour cells with specific fluorescence of catecholamines can be seen surrounded with strands of non fluorescent connective tissue 100 \times

DISCUSSION

The patient described here had a malignant carotid body tumour with regional lymph node metastases. The tumour contained large amounts of catecholamines in the cytoplasm. Doubts have been risen about the existence of malignant forms of this tumour but well documented cases have been reported in the literature (Moberg 1961 Reese *et al* 1963 Pryse Davies *et al* 1964). Most of them have metastasized to the cervical lymph nodes but nearly half of them had distant metastases.

It has earlier been possible to demonstrate large amounts of catecholamines in benign carotid body tumours (Berdal *et al* 1962 Glenner *et al* 1962 Pryse Davies *et al* 1964). In two instances (Glenner *et al* 1962 Pryse Davies *et al* 1964) the catecholamine present was almost exclusively noradrenaline and it may be suggested that the present catecholamine is also noradrenaline as this catecholamine was excreted in the urine. It may thus also be possible that noradrenaline was released from the tumour and this may have caused the patient's hypertension. Such a release can also explain the fatty change of the liver as changes of this type can be experimentally induced by catecholamines (Carlsson & Sjödahl 1963).

The normal human carotid body contains two types of cells with monoamines, one with a yellow green fluorescence probably containing noradrenaline and the other type with a yellow fluorescence containing 5 hydroxytryptamine (Hamberger *et al* 1966). It seems highly probable that the tumour cells in the described case originate from the cells normally containing noradrenaline.

SUMMARY

A malignant carotid body tumour with regional lymph node metastases is described. The tumour contained large amounts of catecholamines. It is suggested that the tumour cells originate from the catecholamine-containing cells in the carotid body.

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FURTHER STUDIES ON THE FINE STRUCTURE OF RENAL TUBULES IN HEALTHY HUMANS

*With a Note on the Effect of the Intravenous Infusion of Low
Molecular Weight Dextran*

By

JAN I. I. ERICSSON GRNELL ANDERS ANDERS BERGSTRAND
HÅKAN BUCHT and PER ÅKE ÖRSTEN

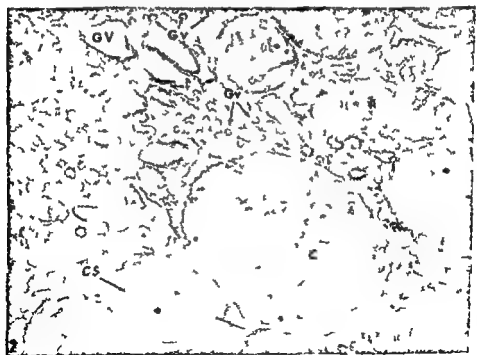
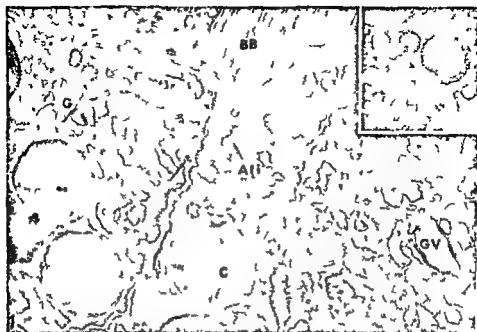
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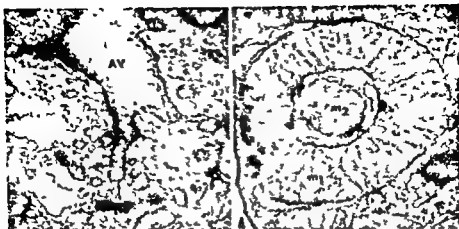
In a previous investigation (Ericsson *et al.* 1966a), we reported on some light and electron microscopic features of renal tubular cells in needle biopsies from young and healthy humans. It was shown that certain differences and modulations of appearance of tubular cells exist between man and experimental animals. It appeared that some of these differences were due to a less satisfactory fixation of the human material—which was obtained through percutaneous needle biopsy—is compared to the renal tissue from experimental animals which was fixed *in vivo* by dripping (Ericsson 1964) or through arterial perfusion (Ericsson 1966a). It is apparent that those discrepancies in structure which are related to the mode of application of the fixative solution cannot be overcome since renal tissue from living humans can only be obtained either through needle biopsy or open surgery. Both of these methods seem to produce approximately the same degree and types of artifacts.

The technique of embedding in methacrylate which was used in our first study (Ericsson *et al.* 1966a) did not permit a detailed evaluation of the fine structure of the various cytoplasmic organelles and also made this material insufficient in some respects as a basis for studies of renal tubule lesions in human material.

It was therefore considered necessary to continue our studies of the normal anatomy of the tubular epithelium in man in order to further elucidate the amount and variations of unavoidable artifacts due to the biopsy technique and to study further the fine structure of the cell

The authors wish to gratefully acknowledge the technical assistance of Miss Cecilia Thes and Mr. Lars Norman in the electron microscope and cytochemical studies.





Figs 3 & 4

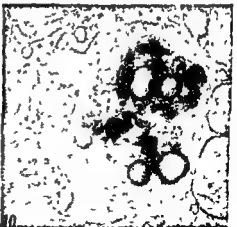
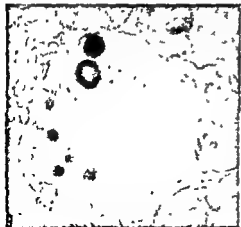
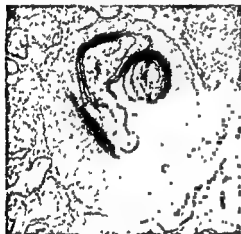
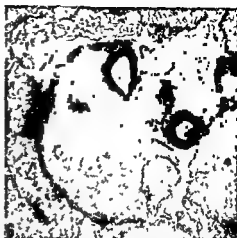
- Fig 3** Control proximal convoluted tubule cell with apical vacuole (AV) in which the "unit membrane" structure of the bordering membrane is clearly visible (arrow). Vestigial leaflets of side and urinary acetate $\times 66,000$
- Fig 4** Control proximal convoluted tubule cell containing a circular probably doughnut shaped mitochondrion (m_1) in the section completely surrounding an other smaller mitochondrion (m_2) embedding and staining as in Fig 3 $\times 44,600$

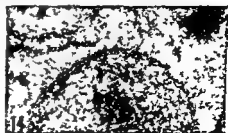
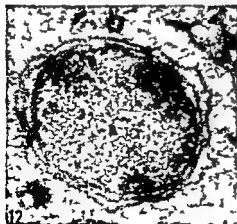
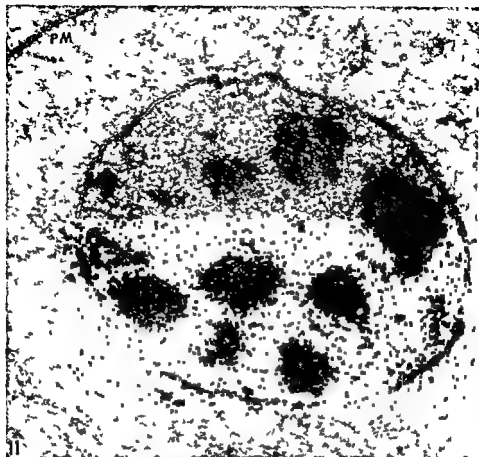
organelles using recent developments in fixation embedding and staining techniques for electron microscopy.

In the present investigation of presumably normal tubules the fine structure of tissues from biopsies processed for optimal preservation of the morphology will be described. As part of an investigation of the functional and morphologic effects of low molecular weight dextran (Rheomacrodex Pharmacia Uppsala Sweden) on the kidney a report of the effect of the intravenous infusion of Rheomacrodex on renal tubule fine structure is included.

Figs 1 & 2

- Fig 1** Control apical portions of proximal convoluted tubule cells. Note the "canted" apical tubular invagination (At) and the irregularly widened extracellular spaces between lateral plasma membranes. In one of the latter spaces is some electron dense material (arrow) present. Inset shows thick matings in the luminal debris 4 apical vesicles of variable size. The matrix of the cytoplasm is fairly dense. BB brush border. C Cili apparatus. CV Colgi vac. Lipon lead hydr. xide $\times 73,000$ inset $\times 45,000$
- Fig 2** Control portion of cytoplasm in proximal convoluted tubule cell with a large cytoplasmic and a cisterna (C) located close to the colgi apparatus. The latter is composed of vacuoles (CV) and vesicles (V). Some of the vacuoles are closely related to the cytoplasm. Note the double layered envelope of the cytoplasmic membrane which contains a well recognizable mitochondrion. Lipon lead hydr. xide $\times 37,000$





tween 30 and 45 minutes. Prior to thin sectioning approximately 1 μ thick sections cut with glass knives on LKB 1 ultratomes were stained with alkaline toluidine blue and were studied in the light microscope. Peripheral areas of the blocks showing good preservation—as judged by light microscopy—were selected for fine structural observations. The thin sections were picked up on formvar and carbon coated grids and were stained with lead hydroxide (Karnovsky 1961) and/or aqueous uranyl acetate (Watson 1955). They were studied in a Siemens Elmiskop I electron microscope.

For paraffin embedding and light microscopy portions of biopsy specimens were fixed in 2 per cent paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Sections of paraffin embedded material were stained with hematoxylin and eosin, van Gieson's connective tissue stain, and the periodic acid Schiff (PAS) method.

OBSERVATIONS

Normal Subjects

A. Light microscopy. The tissues embedded in paraffin showed an appearance similar to that described previously. Thus, many proximal convoluted tubules contained large amounts of "debris" while others showed collapse or formation of apical "blebs". In 1 μ thick sections of plastic-embedded material stained with toluidine blue, expanded basilar extracellular spaces were sometimes observed. However, these structural modulations were not nearly as pronounced as those observed in the previous investigation (Ericsson *et al.* 1965a). Furthermore, artifactual displacement of cellular organelles to tubular lumens, tubular collapse, and general distortion of the tubules was much more infrequent and less pronounced in the present material.

Similar to the situation in the rat kidney, reaction product in the procedure for acid phosphatase showed a distinct localization in intracellular droplet form in all portions of the tubules except some areas

Figures 11-14

Figures 11 through 14 illustrate the appearance—at high magnification—of different cytoplasmic organelles in control cases embedded in Vestopal. With this embedding medium the substructure of bordering membranes was better resolved than with other media. Sections are stained with lead hydroxide and uranyl acetate.

Fig 11 Cytosome containing clumps of dense material and bordered by a triple-layered approximately 100 Å thick membrane. The plasma membrane (1 M) is similar in thickness and appearance as the cytosomal membrane. $\times 210,000$.

Fig 12 A cytoplasmic body (B) possibly a mitochondrion is completely surrounded by two triple-layered approximately 70 Å thick membranes which may represent smooth surfaced endoplasmic reticulum. $\times 165,000$.

Fig 13 Portion of endoplasmic reticulum in one part studied with ribosomes (arrows). The triple-layered bordering membrane is 60-70 Å thick. $\times 230,000$.

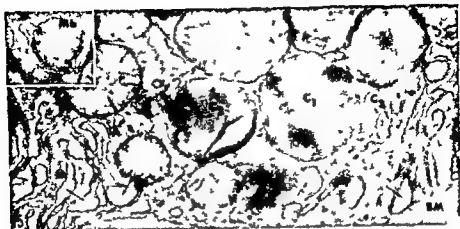
Fig 14 Portion of microbody like structure (31b) bordered by triple-layered 60-70 Å thick membrane. Comparison with the cytosome in Fig 11 shows that the cytosomal membrane is thicker than that of the microbody like element (the magnification is higher in Fig 14). $\times 270,000$.

of the thin limbs of Henle. The droplets were up to approximately $2\ \mu$ in diameter in the proximal convoluted tubules (Fig. 17) while they were smaller and less numerous in the "*pars recta*", (Fig. 18) and the distal portions of the nephron.

B. Electron microscopy. Since some of the features of the tubular epithelium has been described previously (Ericsson *et al.* 1965a), only those structures and modulations of appearance which were not revealed previously will be considered in the following. The localization of acid phosphatase was essentially similar to that in the rat kidney (Ericsson 1966b) with reaction product mainly confined to cytosomes. A more detailed account of the localization of acid phosphatase in the various portions of the nephron will be published elsewhere (Ericsson & Trump 1967), and the results are only summarized here.

Proximal convoluted tubules. The appearance of these tubules is illustrated in Figs. 1 to 15. It was noted that the apical tubular invaginations (Fig. 1) and apical vesicles (cf. Fig. 22) were "coated" in a similar fashion as in corresponding cells in the rat kidney (Ericsson 1964, 1965). These structures seem to participate in pinocytosis, particularly in the transport of proteins and other colloidal compounds (Ericsson 1964, 1965, Roth & Porter 1962). Apical vacuoles (Fig. 3) and vesicles were bordered by a "unit membrane" with similar dimensions as the apical plasma membrane. This supports the assumption (Ericsson 1964, 1965) that these structures represent pinocytosis vesicles and vacuoles which have been pinched off from invaginated portions of the apical cell membrane. Mitochondria varied greatly in size and appearance (Fig. 4) and contained numerous large matrical granules (cf. Fig. 23).

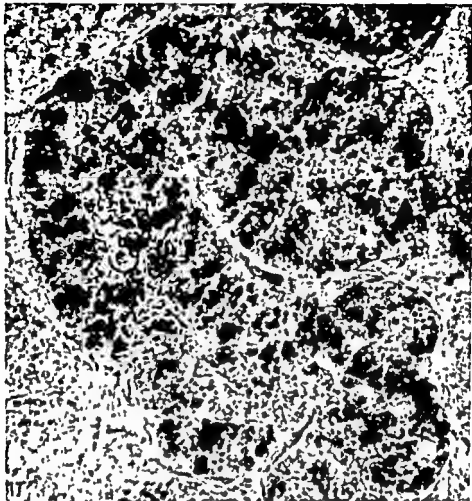
Since single membrane limited cytoplasmic bodies—presumed to represent "lysosomes"—appear to be altered following infusion of dextran and other polysaccharides to experimental animals (Janigan & Santamaria 1961, Maunbach *et al.* 1962, Trump & Janigan 1962) special emphasis was placed on the elucidation of the appearance of such bodies. At least three different types were observed. The most frequently encountered of these bodies resembled the cytosomes in rat kidney proximal tubule cells (Ericsson 1964, Ericsson & Trump 1964) (Figs. 1, 7, and 9, 11). They appeared to contain acid phosphatase (Fig. 19) and were limited by an approximately 100 Å thick, triple-layered membrane. Occasionally, bodies containing clearly recognizable cytoplasmic organelles, such as mitochondria, were seen. Since these bodies were observed so infrequently, it could not be demonstrated with certainty whether they too carried acid phosphatase. Some of them were surrounded by two membranes (Fig. 2), each with a thickness corresponding to that of the endoplasmic reticulum (Fig. 12, cf. Fig. 13). Gradual transitions between such "cytosomes" (Ericsson 1964, Ericsson & Trump 1964, Ericsson *et al.* 1965b) ("autophagic vacuoles") and cytosomes were encountered (Figs. 7 and 8). The third type of



Figs 15 16

Fig 15 Control Base of proximal convoluted tubule cell showing irregular widening of the extracellular spaces (arrows) between the infolded basilar plasma membranes. Three cytosomes (C_1 , C_2 and C_3) are present in the cytoplasm. C_1 lacks membrane inclusions in the matrix. C_2 contains small amounts of such material while C_3 appears to contain cytoplasmic matrix and may correspond to a cytosome. Inset shows a body (Mb) presumed to represent a microbody. It is surrounded by endoplasmic reticulum but—in the plane of the section—does not show presence of a nucleolus. BM basement membrane. Epon Lead hydroxide $\times 24\,000$ inset $\times 29\,000$.

Fig 16 Control Portion of a loop of Henle with flattened epithelium. The apical plasma membrane forms irregular protrusions and microvilli (Mv). Note the very thick and lamellated basement membrane (BM). Note also the filaments (F) in the cytoplasmic matrix. A lipofuscin-like body is present in the cytoplasm. G Golgi apparatus. L tubular lumen. Epon Lead hydroxide $\times 18\,000$.



cytoplasmic body had a finely granular matrix substance was frequently surrounded by endoplasmic reticulum and often contained a slender peripheral rod or plate. Such bodies are illustrated in Figs 11, 15, 22 and 23. These structures have tentatively been referred to as microbodies due to: (a) the close and frequent association with the endoplasmic reticulum (Ericsson 1964; Ericsson & Trump 1966; Voutilainen & Shinn 1964; Trump *et al.* 1962); (b) the similarity of the rods to "nucleoids" in some species (McClure 1964; Jones & Laurell 1966; Trump *et al.* 1962); and (c) the fact that the bodies were limited by an approximately 70 Å thick triple layered membrane (Fig. 14) (Ericsson & Trump 1966; Mannsack 1966).

The Golgi zones were large (Figs 1 and 2 of Fig. 22) and were often closely associated with lysosomes and cytoplasts (Fig. 2).

In the previous study (Ericsson *et al.* 1963a) pronounced widening of the extracellular basilar "compartments" was frequently observed. Although such widening did occur also in the present material it was usually not very marked (Fig. 1 of Fig. 23). Occasionally electron dense "proteinaceous" material was observed in the widened extracellular spaces (Fig. 1).

Straight part of proximal tubules. A few observations were made of this portion of the nephron. The tubular epithelium was low (Fig. 20) with relatively sparse cytoplasmic organelles and small lysosomes. Invaginations of the basilar plasma membrane were either lacking completely or were very shallow.

Other portions of the nephron. The loops of Henle were surrounded by extremely thick and lamellated basement membranes (Fig. 16). The cytoplasm of the lining cells often contained numerous dense lipofuscin-like granules and numerous filaments were disposed in the cytoplasmic matrix.

Two characteristic features of the cells in the collecting ducts were noted: (a) The presence of numerous randomly oriented filaments in the cytoplasmic matrix and (b) peculiar configurations of mitochondrial cristae (Fig. 21).

Figs 17-19

Figures 17 through 19 show the localization of acid phosphatase in a control case.

Fig. 17. Light micrograph showing proximal convoluted tubules with reaction product in "islets" of variable size. $\times 640$.

Fig. 18. Light microscopic picture of pars recta containing numerous small droplets. $\times 480$.

Fig. 19. Electron micrograph showing reaction product mainly on filaments with the size corresponding to that of cytoplasmic filaments. There is some peroxisomal reaction in the cytoplasm surrounding the filaments. Papanicolaou stain. $\times 2400$.

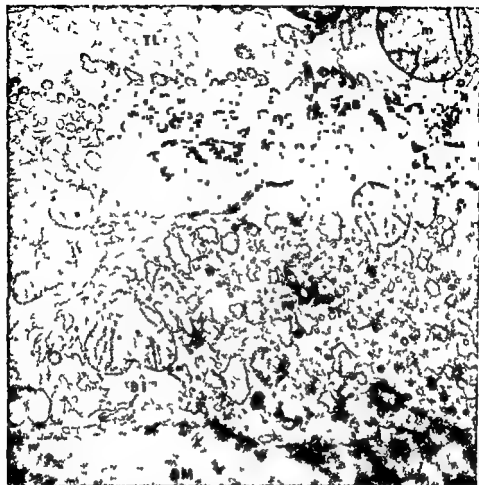


Fig. 20

Central Section from the inner cortex showing the appearance of the straight part of the proximal tubule (para recta). Cyt. plasmic organelles are more sparse the brush border (BB) is lower and the basilar invaginations (BI) of the plasma membrane are more shallow than in the proximal convoluted tubules. There is artifactual localization of a mitochondrion (m) in the lumen of the tubule (TI). BM basement membrane. Epon Leal hydroxide $\times 21,000$.

Subjects Given Rheomacrodex Infusion

A Light microscopy The appearance of the proximal convoluted tubules as well as other portions of the nephron was similar to that in the "controls".

B Electron microscopy Examples of the appearance of proximal convoluted tubules are given in Figs. 22 and 23. With the exception of the occurrence of occasional single membrane limited bodies resembling "absorption droplets" (Ericsson 1965; Maunsbach et al. 1962; Trump & Janigan 1962) and containing a finely granular substance, no alterations clearly at variance with the appearance in the controls were ob-

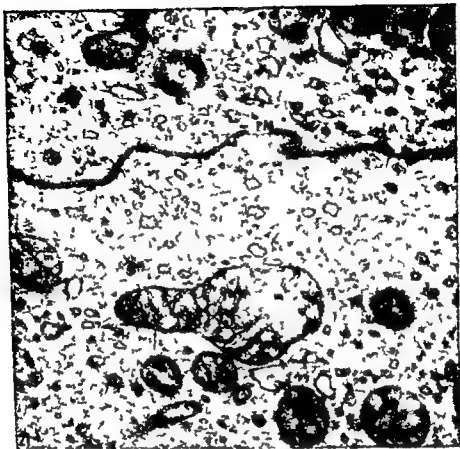


Fig. 21

Control Section from the outer medulla showing portions of two cells in a collecting duct. The cytoplasmic matrix contains abundant haphazardly arranged filaments visible only between envelope and intracistal space (arrows) in the mitochondria (m) and the angular configuration and branching of cristae in the large mitochondrion in the center. C, centro-membrane; pm, plasma membrane; Ep, epithelial hydroxide $\times 36,000$.

served. Although occasional exocytosomes were encountered (Fig. 22) these were not more frequent than in controls.

COMMENT

The observations in this study have in large part corroborated previous results concerning the fine structure of the renal proximal tubules in young and healthy human subjects (Ericsson *et al.* 1967). The improved preservation afforded in the present investigation made it possible to extend the early observations and to study in greater detail various types of cytoplasmic bodies and matrix materials as well as plasma membrane configurations.

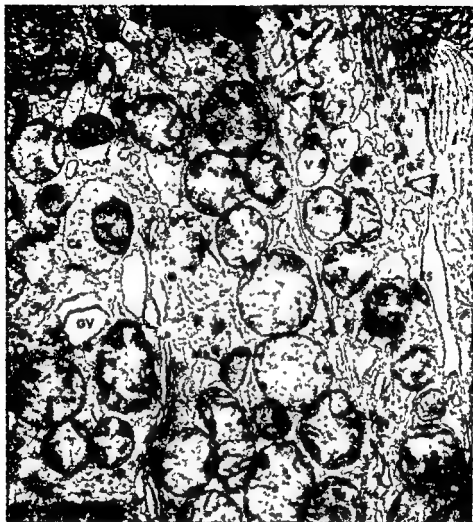


Fig. 22

Rhes macrodex infusion 24 hours prior to biopsy. Proximal convoluted tubule. With the exception of the presence of a single membrane limited "droplet" (Dr) containing a finely granular material, the apical cytoplasm shows the same appearance as in the controls. The vacuoles (V) immediately below the brush border (BV) may represent cytosomes since they contain some membranous material. Two cytoplasmic bodies (Cyb) are closely surrounded by endoplasmic reticulum and contain a slender rod. These bodies may be the equivalent of microbodies in other tissues. V, apical vacuoles; V_s, apical vesicle (with "coating" on the inner surface); C, cytosome containing some membranes material (arrow); CS, cytosegresome; FS, dilated extracellular space between lateral plasma membranes; G and G₁, Golgi zones; G is very large and contains expanded vacuoles (GV); Jc, junctional complex. Epon. Lead hydroxide. $\times 15,000$.

The finding of cytosegresomes in proximal convoluted tubule cells indicates that a physiologic turnover of cytoplasmic organelles occurs in a fashion similar to that in corresponding cells of rat kidney (Ericsson *et al.* 1967b) as well as several other cell types. Although the final

proof that these organelles contain hydrolytic enzymes would require a positive outcome with ultrastructural cytochemical methods the appearance of the bodies makes it highly probable that they are involved in the degradation of the enclosed organelles. The occurrence of apparent transitional forms between cytolysosomes and cytosomes is compatible with the notion (Ericsson 1964; Ericsson *et al.* 1965b) that some cytosomes are derived from cytolysosomes. The cytolysosomes appeared to be formed through wrapping of membranes—with dimensions similar to those of the endoplasmic reticulum—around the organelles set aside for digestion.

The microbodies in hepatic and renal tubule cells of various species appear to contain enzymes other than the hydrolases presumed to be present in cytosomes and cytolysosomes (Afzelius 1965; Baudhuin *et al.* 1965; de Duve & Baudhuin 1966; Hruban & Swift 1964; Tsukada *et al.* 1966). Comparisons of the appearance of microbodies in different species demonstrate that these bodies may show rather marked variations in structure (Afzelius 1965; Ericsson & Trump 1966; Jones & Lawcell 1966; Trump *et al.* 1962) however they are characteristically surrounded by endoplasmic reticulum (Ericsson 1964; Ericsson & Trump 1966; Notikoff, and Shin 1964; Trump & Ericsson 1964) and—in many instances—contain a crystalline “nucleoid” (Afzelius 1965). In rat kidney proximal tubules microbodies are limited by an approximately 70 Å thick triple layered membrane (Ericsson & Trump 1966; Maunsbach 1966). A distinguishing cytochemical characteristic is furthermore their apparent lack of acid phosphatase activity (Ericsson 1964; Ericsson & Trump 1966; Miller & Palade 1964; Trump & Ericsson 1964). Since the bodies observed in the present investigation were closely surrounded by endoplasmic reticulum, were limited by a 70 Å thick membrane and some of them appeared to contain a rod shaped or plate like nucleoid they were tentatively referred to as microbodies. Similar structures have been noted in normal human proximal tubules by others (Bulger & Trump 1966; Tisher *et al.* 1966). The significance of the nucleoid is not clear. In hepatic parenchymal cell microbodies of the rat the nucleoid has a crystalline appearance and may represent uricase (Baudhuin *et al.* 1965; Hruban & Swift 1964; Tsukada *et al.* 1966). However a suggestive crystalline appearance of the nucleoid has also been observed in renal proximal tubules in rat kidney (Ericsson & Trump 1966) where uricase appears to be absent (Graham & Karnovsky 1965; Straus 1966). A non-crystalline nucleoid somewhat similar to the one noted in the present investigation has been described in microbodies of hamster (Jones & Lawcell 1966). Since man—like uricotel vertebrates such as birds and most reptiles—have uric acid as the principal end product in nitrogen metabolism and lack uricase (for reviews see Afzelius 1965 and Florkin & Duchateau 1943) it is unlikely that the rod or nucleoid observed in the present study represents



Fig. 93

After macrophage infusion 24 hours prior to biopsy. Proximal convoluted tubule. With the exception of the presence of a single membrane limited "rosette" (Dr) containing a finely granular material the apical cytoplasm shows the same appearance as in the controls. The vacuoles (V) immediately below the brush border (BB) may represent cytosomes since they contain some membranous material. Two cytoplasmic bodies (M) are closely surrounded by endoplasmic reticulum and contain a slender rod. These bodies may be the equivalent of microbodies in other tissues. Vv, apical vacuoles. Vv, apical vesicle (with "coating" on the inner surface). C, cytosome containing some membranes material (arrow). FS, filate extracellular space between lateral plasma membranes (C and G). Golgi cisternae (G) is very large and contains expanded vacuoles (GV). Jc, junctional complex. Epon. Lead. 15 hr. $\times 15,000$.

The finding of cytosomes in proximal convoluted tubule cells indicates that a physiologic turnover of cytoplasmic organelles occurs in a fashion similar to that in corresponding cells of rat kidney (Friesen *et al.* 1965b) as well as several other cell types. Although the final

uricase. Furthermore, it does not appear to show the characteristic crystalline appearance of uricase (Tsukada *et al* 1966).

Although widened extracellular compartments in proximal convoluted tubules were noted in the present study they were not as large and frequent as was observed previously. It is not likely that this discrepancy is related to the embedding procedure (Fricsson *et al* 1966). It would rather seem that the more rapid penetration of the tissues of a collidine buffered (Bennett & Luft 1959)—as opposed to veronal acetate (Palade 1952) or phosphate buffered (Mallory 1961)— OsO_4 gives a more uniform and reliable fixation (Fricsson & Alalakin 1965; Orrenius & Fricsson 1966). In the rat kidney, widened extracellular compartments in proximal convoluted tubule cells are particularly prominent in a zone subjacent to the one fixed *in vivo* by dripping the fixative onto the surface of the kidney, and also in the zone below the well fixed peripheral portion of tissues fixed by immersion (Fricsson & Riberfeld 1966; Maunsbach *et al* 1962b; Trump & Fricsson 1965). Widening of extracellular compartments can be prevented by perfusion fixation in the experimental animal (Fricsson 1966a). It appears to be related to the tonicity of the fixative solution as well as to increased interstitial pressure following cessation of proper circulatory conditions in the tissues (Maunsbach *et al* 1962b; Trump & Fricsson 1965).

The nature and significance of cytoplasmic filaments is obscure. They have been observed in a large number of different cell types. Their morphologic appearance suggests a supportive function; they may also be associated with cytoplasmic streaming. The occurrence of numerous filaments in the cells of the collecting ducts is compatible with either of these interj relations.

Studies on experimental animals have suggested that various carbohydrates and polysaccharides which are filtered through the glomeruli are reabsorbed in the proximal tubules, probably by way of pinocytosis (Gloor 1960; Maunsbach *et al* 1962; Trump & Jantzen 1962). It appears furthermore that these substances are transported to and stored within the cytosomes. If the absorption is excessive the cytosomes become greatly enlarged and the light microscopic picture of "vacuolar nephropathy," "osmotic nephrosis" or "hydropic degeneration" ensues.

DISCUSSION

Rheomacrodex infusion 24 hours prior to III 155. Base of proximal convoluted tubule cell showing normal appearance of cytoplasmic constituents and matrix substance. Note focal expansions (arrows) of extracellular spaces between infolled basilar

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NOTE ADDED IN PROOF

After this paper was submitted for publication a paper by *Tisher et al* (Lab Invest 15: 1357 1966) has been published describing the fine structure of the human proximal tubule. The findings are essentially in agreement with our observations. Thus the authors describe the occurrence of microbodies with rod shaped or plate formed nucleoli and they show several pictures of cytosegresomes.

Plateformed nucleoli in microbodies of bovine liver were recently described by *Shafitka* (J Ultrastr Res 16: 603 1966).

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TROPHOBLASTIC AND SUBTROPHOBLASTIC MINERAL SALT DEPOSITION IN HYDRAMNIOS

By

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Received 22 vi 66

In a previous paper, *Ivemark et al* (1962) reported two cases of generalized arterial calcification and hydramnios in stillborn infants. Both cases showed deposition of hydroxyapatite in the elastic layer of large arteries and in the trophoblastic layer of the placental villi. This particular type of salt deposition in the placenta is to be distinguished from the usual type of calcification in the ageing placenta, since the latter type mainly affects the stroma of degenerating placental villi.

The present study was undertaken in order to find out whether trophoblastic salt deposition is a common feature of hydramnios.

MATERIAL AND METHODS

Twenty-four placentas were consecutively collected from cases of verified hydramnios. Ninety placentas from non-hydramniotic pregnancies served as a control group.

The hydramniotic group included one case from the 2nd and 23 cases from the 3rd trimester of pregnancy. In eight cases there were congenital malformations, in three of these the malformation involved the digestive tract (oesophageal atresia, cheilognathopalatoschisis, agenesis of the upper digestive tract in a monster). Five other cases displayed isolated or combined cardiovascular, pulmonary or cerebral malformations. Maternal diabetes was present in two, toxæmia of pregnancy in four cases. There was no instance of iso-immunization. Fifteen of the infants were stillborn.

In the control group fifty of the placentas were consecutively collected from uncomplicated full-term pregnancies, the placentas as well as the infants being considered grossly normal. The remaining forty placentas were consecutively collected from cases of perinatal death. Two of these were from the 2nd and 38 from the 3rd trimester of pregnancy. In cases of various types including one case involving malformations involve the digestive tract and iso-immunization in eight cases. The

For histologic examination of the placenta blocks were taken from two to five areas, fixed in 10 per cent neutral formalin and embedded in paraffin. Sections 3-5 μ thick from these blocks were stained with haematoxylin-eosin (PAS), von Kossa stain for phosphates and carbonates and with the Murexide method, which according to Kaufman & Adams (1957) is highly specific for calcium. In addition sections were stained with Prussian blue for iron, since sub-trophoblastic iron deposition has been reported to occur during the second trimester of gestation (*McKay et al* 1958).

Presence of trophoblastic and subtrophoblastic deposition of calcium, iron and phosphates/carbonates was recorded separately. The amount of such deposition in each case was graded as follows:

0	No deposition
+	Deposition in less than 10 per cent of the villi
++	Deposition in 10-30 per cent of the villi
+++	Deposition in more than 30 per cent of the villi

For microradiographic examination 5 μ thick sections were cut from the formalin fixed specimens and placed in close contact with a fine grain photographic emulsion. The sections were exposed in a fine focus X-ray tube at the wave length range 8-20 Å. The microradiograms of the placentas were compared with the corresponding stained sections.

For evaluation of the statistical significance of the results Yeats' corrected χ^2 -test was used.

RESULTS

Incidence and Degree of Mineral Deposition

Trophoblastic and subtrophoblastic salt deposition was found in nineteen of the twenty-four hydramniotic cases (79 per cent). Fourteen of these displayed deposition of calcium, iron and phosphate/carbonate. Three cases showed iron deposition only. In the remaining two cases there was deposition of calcium and phosphate/carbonate but not of iron.

In the control group no trophoblastic or subtrophoblastic deposition of calcium or phosphate/carbonate could be demonstrated. In five cases, all from the 3rd trimester (5.5 per cent), subtrophoblastic iron deposition was found.

TABLE 1

The Occurrence of Various Grades of Trophoblastic Mineral Deposition in Twenty Four Cases of Hydramnios

Grade*	Per cent of cases with deposition of		
	Calcium	Iron	Phosphate Carbonate
0	25	29	33
+	21	17	21
++	38	33	33
+++	8	21	8

* For definition see text

The degree of salt deposition in the hydramniotic group and in the control group is presented in Tables 1-2. The difference between hydramniotic and non hydramniotic groups as regards presence of trophoblastic salt depositions is highly significant for both calcium and iron as well as for phosphate/carbonate ($p < 0.001$). No correlation could be found between the occurrence of such mineral deposition and malformations of the foetus or obstetric complications (stillbirth, toxæmia of pregnancy).

TABLE 2

*The Occurrence of Various Grades of Trophoblastic Mineral Deposition in Ninety
 Von Hydramniotic Cases (Fifty Placentas from Full Term Uncomplicated
 Pregnancies + Forty Placentas from Cases of Perinatal Death)*

Grade*	Per cent of cases with deposition of		
	Calcium	Iron	Phosphate/Carbonate
II	100	95	100
+	0	3	0
++	0	1	0
+++	0	1	0

* For definition, see text

Histologic Characteristics of Mineral Depositions

The *calcium depositions*, as demonstrated with the Murexide stain, were localized mainly in the cytoplasm and in the intercellular space of the syncytial trophoblast (Figs 1 and 3). Only occasionally were they found in the subtrophoblastic region, in or near the trophoblastic basement membrane.

The distribution of villi with trophoblastic calcification was patchy; in some areas most villi showed calcium deposits and in other areas no villi were affected. In the main, areas with calcification were localized in the subchorionic region of the placenta. This localization was particularly evident in cases with few affected villi. The calcification mainly affected the small terminal and medium sized villi.

In villi with trophoblastic calcium deposition there was stromal oedema and degeneration with fibrinoid changes and some necrosis. Their basement membranes were thickened with PAS positive material (Fig. 2). Villi without evidence of trophoblastic calcium deposition were normal, although cases of foetal death showed some stromal condensation and vascular collapse. Both in affected and unaffected villi the degree of trophoblastic knotting and persistence of cytotrophoblasts was similar.

The localization of the *iron depositions* differed clearly from that of the calcium depositions; iron was found exclusively in the subtrophoblastic region, in and around the trophoblastic basement membrane, as a rule underneath the calcium and phosphate/carbonate depositions (Fig. 3). Villi with iron deposition were distributed irregularly and could be found anywhere in the placenta. Stem villi, medium sized villi and terminal villi were equally affected. Apart from iron deposition the affected villi appeared normal with no evidence of stromal degeneration.

Phosphate/carbonates, as demonstrated with the von Kossa stain, were mainly localized to the same trophoblastic region as the calcium depositions (Fig. 4), but were also encountered in the same region



Figs 1 and 2

- Fig 1* Placental villi with calcium deposition in the trophoblastic layer. Hydramnios (gestational age 6 months). Murexile stain $\times 530$
- Fig 2* Fibrin stromal degeneration and thickening of subtrophoblastic basement membrane areas (placental villi with trophoblastic calcium deposition). Hydramnios, full term pregnancy, placental weight 350 g. PAS $\times 210$

as the iron depositions etc in the subtrophoblastic basement membrane

In placentas from hydramniotic cases showing depositions of calcium iron and phosphates/carbonates the various depositions largely occurred together in the same villi

Microradiographic Findings

The microradiographic examination of the placentas with trophoblastic villus deposits showed radioactivity of these deposits as previously described in two cases (Itemark *et al* 1962). When compared with the staining reaction it was found that this radioactivity was evident both in cases of Murexide positive—Prussian blue negative depositions and Murexide negative—Prussian blue positive depositions. However the radioactivity was heavier in Murexide positive instances.

DISCUSSION

The present study has revealed a high incidence of trophoblastic salt depositions in the placenta of cases with hydramnios whereas in non-hydramniotic instances no such depositions were encountered. To judge from the staining properties of these deposits they contained calcium and phosphate/carbonate. This is in agreement with previous X-ray crystallographic examinations of such deposits in which they were shown to consist of hydroxyapatite (Itemark *et al* 1962).

Subtrophoblastic iron deposition in the normal placenta has been reported to be a feature of the second trimester of pregnancy (McKay *et al* 1958). In our control series of 88 placentas from the third trimester such deposition was seen in 55 per cent. To judge from the significantly higher rate (71 per cent) of this deposition in the placentas from cases of hydramnios all except one from the third trimester iron deposition in hydramnios seems either to persist or to be delayed in formation.

Whether the salt depositions in the placental villi seen in the cases of hydramnios in the present material may be of aetiological significance in the development of hydramnios or are secondary to hydramnios developed on other grounds cannot be assessed. However the deposition may well constitute a morphologic basis for decreased transplacental exchange of water between mother and foetus which has been demonstrated in the hydramniotic state by Hutchinson *et al* (1959).

The degenerative changes in villi with trophoblastic depositions of calcium salts may be due to a decreased transport of nutritive substances through the placental barrier.

SUMMARY

Twenty-four placentas from cases of hydramnios and ninety placentas from non hydramniotic cases were examined histochemically and with microradiography for the occurrence of trophoblastic and subtrophoblastic salt deposition.

Calcium deposition along the trophoblastic layer was present in sixteen of the hydramniotic cases (67 per cent), whereas in the non-hydramniotic cases no such deposition was found. Degenerative changes were observed in the stroma of villi with calcium salt deposits.

Iron deposition along the subtrophoblastic basement membranes was noticed in seventeen of the placentas from cases of hydramnios (71 per cent) and in five of the ninety control cases (5.5 per cent). No degenerative changes were seen in the stroma of villi with iron deposition.

Carbonate/phosphate deposition was generally shown to accompany the calcium, and only rarely the iron deposition. All these depositions corresponded to areas of radiopacity in microradiograms.

It is suggested that the trophoblastic deposits in the placental villi in cases of hydramnios may interfere with the transfer of water and other substances through the placental barrier.

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SEX DIFFERENCES IN TUMOUR GROWTH AND LYMPHOID REACTIONS IN MICE WITH EHRICH'S ASCITES CARCINOMA

By

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Previous investigations have revealed that inhibition of lymphoid reactions by whole body irradiation does not alter early intraperitoneal growth of the Ehrlich ascites carcinoma (IAC) (Thunold 1966 a, b). On the other hand it is well known that both subcutaneous and intraperitoneal growth of homotransplantable tumours cause a general stimulation of the lymphoid tissue though this is usually insufficient to prevent tumour growth (Wogtom 1929, Old *et al* 1959, 1960).

It has been shown that IAC grows better in male than female mice on subcutaneous injection (Hartvelt 1962, 1965) but no sex differences have been demonstrated on intraperitoneal transplantation (Thunold 1966 b). The following experiment was planned to see if sex differences in growth of IAC could be related to morphological changes in the lymphoid system.

MATERIAL AND METHODS

Mice. Adult male and female albino mice of similar age from an outbred but selected line were used (Thunold 1966 a). Five groups of 12 males (mean weight 24.4 ± 0.5 g) and 12 females (mean weight 21.1 ± 0.3 g) were set up.

Tumour. The Ehrlich ascites carcinoma used was a 10 day transplant from a female mouse of the selected line.

Experimental procedure

Intraperitoneal tumour injection. Twelve male and 12 female mice were each given 5.6×10^5 tumour cells in 0.1 ml whole tumour ascites intraperitoneally.

Subcutaneous tumour injection. A second group of male and female mice received the same tumour intrasubcutaneously in the back.

Seven days after transplantation all tumour-bearing mice were killed by cervical dislocation. The animals were weighed to the nearest 0.5 g after removal of intraperitoneal and subcutaneous tumours respectively. The total packed tumour cell volume and the cell surface volume were determined for the intraperitoneal tumours as described previously (Thunold 1966 a). Films were made at once from the tumour as they were dried and stained with Leishman's stain. Two hundred tumour cells were counted and the number of large lymphoid cells expressed as a percentage of each group. The ulceraneous tumours were removed and weighed wet weight. The viscera were formalin fixed and stained with haematoxylin and eosin for histological examination. The pleural and liver viscera were removed and weighed to the nearest 0.1 g. Lymph nodes were taken from the mesentery and from the neck region in mice carrying intraperitoneal and ulceraneous tumours respectively. The

lymph nodes were not weighed. Sections from all organs were stained with haematoxylin and eosin and methyl green pyronin and examined microscopically.

Control test for stimulating effect on the lymphoid system A minced suspension of liver and spleen tissue from closed colony female mice was prepared in 0.8% per cent saline and 0.1 ml injected intraperitoneally to 12 males and 12 females. In addition a Seitz filtrate of the ascitic fluid from the tumour bearing donor mouse was prepared and 0.1 ml injected intraperitoneally into the 4th group of male and female mice. These animals were also killed on the 7th day after injection, the spleens and livers were removed, weighed and stained for histological examination as stated above.

Normal organ weight and histology The 5th group of mice were killed on the same day, and the spleens and livers were weighed and examined histologically. Some mesenteric and subcutaneous lymph nodes were removed and examined in stained sections.

Bacteriological examination The tumours were treated under sterile conditions. Aerobic cultures from the inoculum and some of the intraperitoneal and subcutaneous transplants were made on blood agar and serum broth. For anaerobic incubation the same media plus a 10% glycolate medium (Haacke and Thio glycolate Medium) were used.

RESULTS

Tumour growth and microscopy As shown in Table 1 there was no sex difference in intraperitoneal tumour growth, while a clear difference was demonstrable on subcutaneous transplantation as the tumours in the male mice were larger than in the females ($P < 0.01$).

TABLE 1

The Intraperitoneal (IP) Packed Tumour Cell Volume (PCV) the Cell Free Fluid Volume and the Subcutaneous (SC) Tumour Weight in Male and Female Mice 7 Days after Injection of the Ehrlich Ascites Carcinoma. Entries means \pm SD

Route of injection	No. and sex of animals	PCV of tumour cells (ml)	Cell free fluid (ml)	SC tumours (mg)
IP	12 σ	1.65 ± 0.32	3.28 ± 1.21	
	12 ϕ	1.24 ± 0.29	3.70 ± 1.16	
SC	12 σ			154.9 ± 67.4
	12 ϕ			67.8 ± 44.4

Cytological investigation of the intraperitoneal tumours revealed 1 per cent and 6 per cent large atypical tumour cells in male and female mice respectively. These cells were similar to those previously found in non-irradiated mice with IAC (Thunold 1966 a). The sex difference was not significant. Similarly histological investigation of the subcutaneous tumours showed no sex difference as some tumours from mice of both sexes showed small central areas with haemorrhage and necrosis.

Organ weights The increase in mean spleen and liver weight following intraperitoneal and subcutaneous tumour growth is demonstrated in Fig. 1. The absolute weights were used as no significant body weight change occurred during the experimental period. On intraperitoneal tumour growth a significant increase in both spleen and

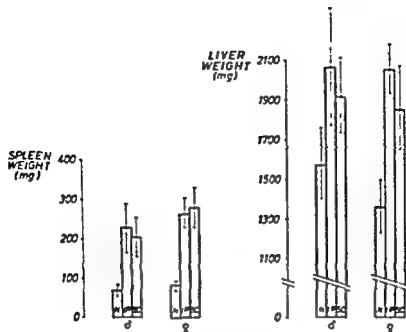


Fig. 1

Spleen and liver weight in male and female contr. 1 mice (N) and 7 days after intra-peritoneal (IP) and subcutaneous (SC) injection of Ehrlich's ascites tumour. Entries: means (mg) \pm SD.

liver weight can be seen in male ($P < 0.001$) and female ($P < 0.001$) mice. On subcutaneous tumour growth the increase in spleen weight was significant in both males ($P < 0.05$) and females ($P < 0.001$). The liver weight increase on subcutaneous tumour growth was similar in the two sexes ($P < 0.001$).

TABLE 2

The Average Increase (per cent) in Weight of Spleen and Liver in Mice with Intraperitoneal (IP) and Subcutaneous (SC) Ehrlich's Ascites Carcinoma

Sex of animals	Spleen wt. Increase (%)		Liver wt. Increase (%)	
	IP	SC	IP	SC
♂	22½	1¾	35	35
♀	23½	250	46	36

The average percentage increase in spleen and liver weight is shown in Table 2. In all groups there was a tendency to greater organ weight increase in female than in male mice. Only the sex difference in spleen weight increase following subcutaneous tumour injection was significant ($P < 0.05$).

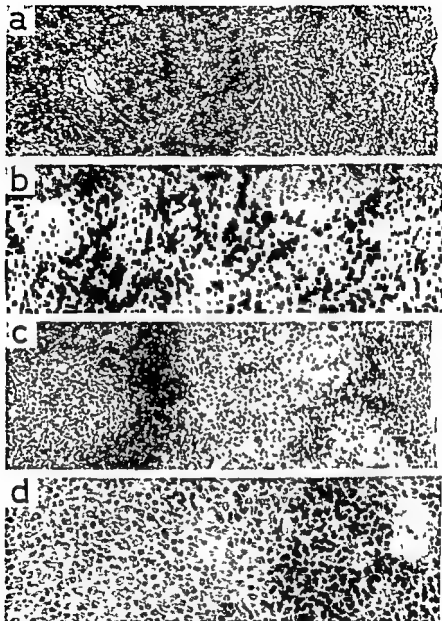


Fig. 2

- a Normal mouse spleen with resting lymphoid follicle (left) and red pulp. Haematoxylin and eosin $\times 150$
- b Normal spleen with no distinct pyroninophilia in either follicle (left) or red pulp. Methyl green pyronin $\times 380$
- c Spleen from tumour bearing mouse showing hyperplasia of the lymphoid follicle (left). The red pulp is cellular and contains a number of erythrocytes. Haematoxylin and eosin $\times 150$
- d Spleen from tumour bearing mouse with large number of pyroninophilic cells in the red pulp (right). Note relative absence of pyroninophilia in the follicle (left). Methyl green pyronin $\times 330$

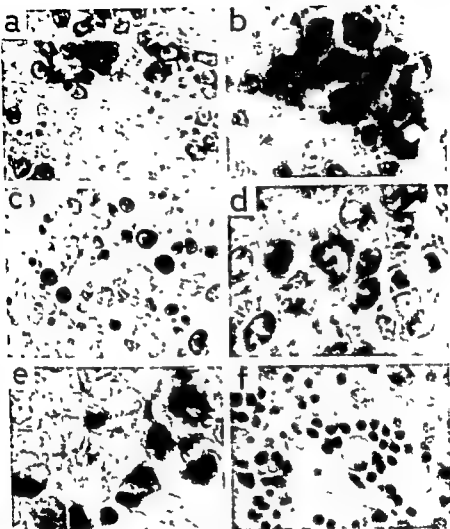
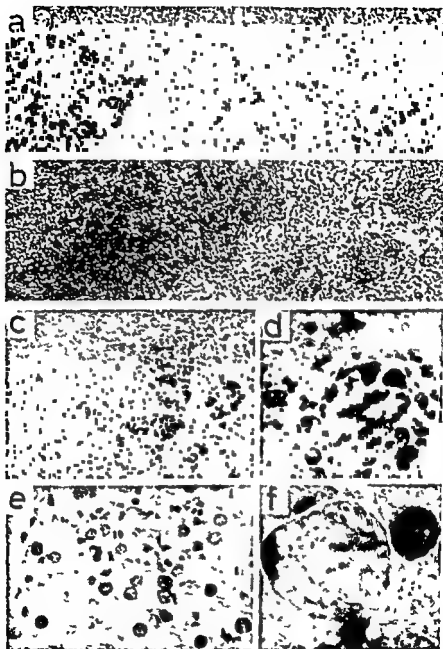


Fig. 3

Spleen from tumour-bearing mouse

- a Red pulp with large angular pyrenocytic cells. These have abundant cytoplasm and prominent nuclei. Methyl green pyronin $\times 610$
- b Greater magnification of pyrenocytic cells $\times 1500$
- c Red pulp with large pyrenocytic cells, plasma cells and some lymphocytes. Methyl green pyronin $\times 610$
- d Cells in mitosis from germinal centre. Methyl green pyronin $\times 1500$
- e Cell in red pulp in division. Methyl green pyronin $\times 1500$
- f Red pulp with large number of erythrocytes with a tendency to clumping (erythrophagocytosis?) and granulocytes. Haemalum and eosin $\times 610$



Organ histology In normal spleens the germinal centres of the Malpighian bodies contained small darkly stained cells with scanty cytoplasm and only a few erythrocytes were present in the red pulp (Fig. 2 a). No distinct pyroninophilus was seen (Fig. 2 b). Occasionally mast cells were present.

Following both *intra-peritoneal* and *subcutaneous* tumour growth the Malpighian bodies in the spleen showed prominent germinal centres (Fig. 2 c). The most remarkable change occurred in the red pulp which became cellular with increased pyroninophilia (Fig. 2 d). These cells were large with prominent nucleoli and abundant pyroninophilic cytoplasm (Fig. 3 a b). Occasionally plasma cells were seen (Fig. 3 c). There was increased mitotic activity in both the germinal centres (Fig. 3 d) and in the red pulp (Fig. 3 e). In addition a large number of erythrocytes with a tendency to clumping (erythrocytopenia?) were seen (Fig. 3 f). There was no obvious changes in number or appearance of mast cells.

A section from a normal lymph node with a resting nodule is shown in Fig. 4 a. On tumour growth the regional lymph nodes were enlarged with large lymphatic nodules with prominent germinal centres and cords of hyperplastic lymphoid tissue could be seen in the medulla (Fig. 4 b). In the latter numbers of large pyroninophilic cells were present (Fig. 4 c d).

Following both *intra-peritoneal* and *subcutaneous* tumour growth red cells and granulocytes were present in the liver sinusoids (Fig. 4 e). A slight increase in the size of the Kupffer cells and erythrocytopenia could be seen (Fig. 4 f).

Microscopy of the organs did not reveal any measurable sex differences.

Extramedullary haematopoiesis In the normal spleen extramedullary haematopoiesis was found regularly in the red pulp. Erythropoietic foci identified by the small darkly stained nuclei (Dunn 1954) were predominant and megakaryocytes were a constant finding. Following tumour growth the erythropoiesis was more intense and the number of megakaryocytes increased (Fig. 5 a b).

Fig. 5

- a Normal mouse lymph node with resting lymphatic nodule (left) Haematoxylin and eosin $\times 150$
- b Lymph node from tumour bearing mouse showing large lymphatic nodule (left) and cords of hyperplastic lymphoid tissue in the medulla (right) Haematoxylin and eosin $\times 15$
- c Lymph node from tumour bearing mouse showing large lymphatic nodule (right) Some mast cells can be seen
- d Greater magnification ($\times 610$) of Fig. 4 c showing erythrocytopenia
- e Liver tissue from tumour bearing mouse showing accumulation of red cells in the sinusoids Haematoxylin and eosin $\times 350$
- f Greater magnification ($\times 1000$) of Fig. 4 f showing erythrocytopenia

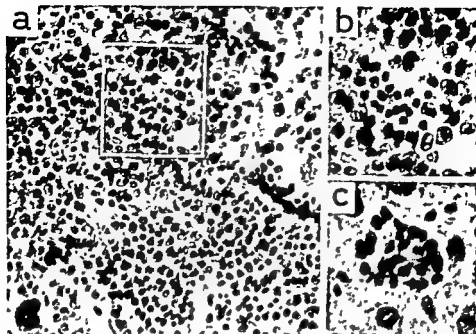


Fig. 5

- a Spleen from tumour bearing mouse. The red pulp is crowded with large cells with pale nuclei and prominent nucleoli, and lymphocytes. Several foci with erythroid elements with dark stained cells and two megakaryocytes can be seen. Haematoxylin and eosin $\times 390$.
- b Greater magnification of the inset in a showing an erythropoietic focus $\times 610$.
- c Liver from tumour bearing mouse showing a sinusoid with granulopoietic elements of different maturity. Haematoxylin and eosin $\times 610$.

Neither normal lymph nodes nor the lymph nodes of tumour bearing animals showed evidence of haematopoietic activity.

Scattered haematopoietic elements were found in the livers of untreated animals. In this organ granulocytopoiesis was preponderant, and megakaryocytes could not be seen. After tumour growth granulocytopoietic elements were found more regularly (Fig. 5 c).

Sex differences in haematopoietic activity were not seen.

Control test on stimulation effect on the lymphoid tissue. Neither normal mouse tissue nor a Scitz filtrate of the tumour ascitic fluid produced any increase in spleen or liver weight or gave any changes in organ histology when investigated 7 days after injection.

DISCUSSION

The present experiment confirms the previous findings that EAC grows better in male than in female mice on subcutaneous transplantation (Hartvelt 1962, 1965), while no sex differences can be demonstrated in intraperitoneal growth. As a free suspension of tumour cells was used as inoculum, the number of cells given to each mouse was the

same. Thus the sex difference in subcutaneous tumour growth is likely to be due to differences in host reaction.

Following tumour growth a significant increase in spleen and liver weight is found in both sexes. The increase in spleen weight was greater in female than in male mice after subcutaneous tumour injection. No sex difference could be demonstrated in either spleen or liver weight increase in the other groups.

The injected tumour was not contaminated with bacteria, and there was no increase in organ weight or change in histology after injection of a cell free Seitz filtrate of the ascitic fluid or a suspension of normal mouse tissue cells. Normal mouse tissue can not be regarded as a valid control in the present experiment. It will not proliferate like tumour cells, and the stimuli can thus not be compared quantitatively, even if successive injections had been given. The organ enlargement may possibly to some extent be due to the increase in extramedullary haematopoiesis, but it seems in any case justifiable to conclude that the hepato-splenomegaly can be taken as an expression of host reaction against the progressively growing tumour cells. It should be stressed that the findings cannot be taken as a reaction to a tumour specific antigen in this tumour host system.

The increase in spleen weight with tumour growth is associated in the present experiment with marked histological changes in the white and red pulp of the spleen. Similar weight and histological changes have been found with other progressively growing tumour homografts (El Hassan & Stuart 1965). These changes in general resemble those seen during non tumour homograft rejection (Scolthorne & McGregor 1955; Gouans 1962; Gouans et al 1963). Thus they may be interpreted as indicating a homograft reaction to antigens present in the TAC. It has been postulated that this tumour grows due to lack of intelligibility (Hauschka & Ames 1957) but it has however, been shown to contain antigens not present in normal mouse tissue (Fasty & Amlrose 1957).

The splenic enlargement is probably partly due to accumulation of erythrocytes. It is not reasonable that this red cell accumulation is due to simple congestion. All mice, the controls included, were killed in the same way, and the phenomena is also seen constantly in mice bearing small subcutaneous tumours. The possibility remains that red cells are trapped in the spleen by means of a non specific filter mechanism (see Pringle 1965) or because of sensitization of the red cells (Janell 1955). Adelsberger & Zimmerman (1954) have found that red blood cells of mice bearing isologous tumours behave similarly in the molting system to mouse erythrocytes sensitized with antitumour red blood cell serum, indicating an immunological change in the erythrocytes during tumour growth. Such a possibility may also exist in the case of the homotransplantable TAC.

The change in lymph node histology during tumour growth is similar

to that found during non-tumour homograft rejection (Ellis *et al* 1950, Scolthorne 1957) and thus gives additional evidence of immune response to the tumour homograft in the present experiment

The origin of the large pyroninophilic cells found in the spleen and lymph nodes has been a matter for discussion (Gowans 1962, Burwell 1962), and it has not been the purpose of the present study to elucidate this particular question. The erythropoietic elements in the spleen seem to be activated during tumour growth, and it may be argued that immature erythroblasts can give a similar pyroninophilic picture. However, the great number of pyroninophilic cells in the lymph nodes where no erythropoietic foci can be found and the diffuse pyroninophilia of the spleen red pulp, suggest that the pyroninophilic cells are mainly an expression of immunological activation.

While spleen and lymph node changes are striking, the histological changes in the liver parenchyma are not so characteristic. It has been shown that the liver plays an important rôle in removing foreign or altered red cells from the blood stream (Jandl & Kaplan 1960, Nelson & Buras 1963). The present finding of erythrophagocytosis in the liver may support the previous suggestion of a change in red cell properties during growth of EAC.

The present experiment thus gives histological evidence of activation of the immune response during both intraperitoneal and subcutaneous growth of EAC. It is of interest to note the direct correlation between smaller subcutaneous tumours and greater per cent increase in spleen size in female compared to male mice. The immunological nature of this stimulation may suggest that the immune response to the tumour has been stronger in female than male mice with consequent inhibition of subcutaneous tumour growth. This is supported by the fact that Andervont (1932) found it easier to induce resistance to sarcoma 180 in female mice. Gross (1911) demonstrated smaller incidence of takes and greater incidence of regressions of sarcoma 37 on intradermal inoculation in female mice, indicating a greater natural resistance in this sex to tumour growth. In addition, it has been shown that the response to stimulating processes evoked by bacterial infection (BCG) is stronger in female than male mice (Halpern *et al* 1960).

On the other hand it has also been shown that increase in spleen size is greater in mice with progressively growing tumours than in those with receding ones (Bashford 1913, Woodruff & Symes 1962), indicating that there is not necessarily any connection between spleen size and tumour resistance. A marked lymphoid reaction can be found however, in the regional lymph nodes to the regressing tumours (Woodruff & Symes 1962) a finding which may confirm that the seat of the reaction which destroys a first-set homograft lies mainly in these nodes (Mitchison 1951). It can thus be argued that the homografted tumours in these experiments (Bashford 1913, Woodruff &

Symes 1962) were destroyed before the spleen was exposed to sufficient antigenic stimulation to show histological evidence of immune activity.

No sex differences were demonstrable in either lymphoid stimulation or tumour growth during intraperitoneal growth. There is no reason to believe that the increased reactivity in the female sex should not work also in the case of intraperitoneal tumour growth. However, intraperitoneal transplants of I AC usually grow fast and kill the hosts rapidly. Thus the immune response of the recipient may easily be overwhelmed and a possible sex difference in reactivity disappear. The finding of a factor in the ascitic fluid of I AC that protects tumour cells from lysis (Harli et 1961) may be of significance in this connection.

These observations may be related to the fact that some non-specific tumours give successful intraperitoneal growth while their ability to grow on subcutaneous or intramuscular transplantation is limited (Sachs & Gallily 1956). I AC seems to grow progressively in one hundred per cent of animals on intraperitoneal injection (Forwenthal & Jahn 1932, Lettré 1941, Thunold 1966 b), while Brodersen (1943) found approximately 81 per cent progressive growth of a solid form. It may thus be concluded that successful homotransplantation even of this highly non-specific tumour is to certain extent dependant on the site of injection and the immune response of the host.

SUMMARY

The growth of Ehrlich's ascites carcinoma (I AC) has been compared in male and female mice of similar age and related to changes in the lympho-reticular system.

On intraperitoneal transplantation no significant sex difference appeared in tumour growth while the tumour grows better in male than female mice following subcutaneous injection.

Following tumour growth a marked hyperplasia of the lymphoid system can be found in both sexes. Thus hyperplasia can be related to histological changes which are of probable immunological nature presumably evoked by tumour antigens. While intraperitoneal tumour growth showed no sex differences in these reactions the sex difference in subcutaneous tumour growth can be directly related to a stronger host reactivity in the female sex.

It is concluded that the immune response may be of importance to subcutaneous growth of I AC. The pertinence of these observations to the fact that some homotransplantable tumours take better on intraperitoneal than subcutaneous or intramuscular injection is discussed.

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EFFECTS OF HETEROSPFCIFIC ANTISERUM AGAINST LYMPH NODE CELLS ON THE DEVELOPMENT OF EXPERIMENTAL AMYLOIDOSIS IN MICE

By

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In assessing the role of the immune apparatus in the pathogenesis of experimental amyloidosis in mice a number of procedures have been employed in order to modify the response of the organism to a long standing antigenic treatment leading to amyloidosis. In these experiments it has thus been shown that cortisone when applied late in the course of amyloidogenic treatment—that is late in or after the initial pyroninophilic phase—will enhance the formation of amyloid (Tellum 1952) while cortisone applied earlier in the experiment will prevent or greatly diminish the formation of amyloid deposits mainly by preventing or postponing the pyroninophilic phase (Christensen 1961). Nitrogen mustard administered after cytoplasmic pyroninophilia of reticular cells has developed will even more markedly enhance amyloid deposition (Tellum 1954). A similar effect has been observed after whole body or spleen x irradiation (Christensen & Hjort 1959, 1960). These experiments lend support to the two-phase cellular theory of local secretion of amyloid originally described by Tellum (1961).

In the field of experimental immunology a modified immune apparatus has been obtained by a variety of procedures such as surgical ablation techniques, ionizing radiation or cytotoxic chemical compounds. As these methods are rather unspecific and often accompanied by undesirable, sometimes fatal side effects, the search for a more selective immune depressant has continued and has in recent years led to the rediscovery of the leukocytotoxic serum originally described by Metchnikoff (1899) and the more specific antilymphocytic antiserum produced by Pappenheimer in 1917. Such antilymphocytic sera seem primarily to suppress immune mechanisms of the cellular or delayed type in which lymphoid cells are known to be the mediators. Recent

observations seem to imply a participation of immune mechanisms akin to the cellular type in the pathogenesis of experimental amyloidosis (Ranlov & Jensen 1966, Ranlov 1966). Further, though a cellular origin of amyloid seems well established (Tetum 1961, Cohen *et al* 1965, Werdelin & Ranlov 1966) the exact origin of such cells and their possible relation to lymphoid cells are not known. For these reasons it was decided to investigate the effects of a heterospecific antiserum against lymph node cells on the development of casein-induced murine amyloidosis.

MATERIAL AND METHODS

Animals. Randomized, inbred C3H mice of equal sex distribution, 2-4 months old.

Antiserum. Albino rabbits weighing 3.0-3.5 kg were immunized once a week for 6 weeks with from 2 to 5×10^6 murine lymph node cells per injection. The cell suspensions were prepared from axillary, inguinal and abdominal lymph nodes obtained from donors of the inbred C3H strain. The lymph nodes were freed from contamination with blood and adjacent tissues, grained once in a loose fitting Potter-Fleuhjem homogenizer, washed 3 times in 10 ml of Ringer's solution and resuspended in 2 ml. After cell counting and viability test with trypan blue which showed 80-90 per cent viable cells the suspension was mixed with an equal volume of Freund's complete adjuvant (Difco) and injected in multiple localizations in the rabbit. These were bled 10-14 days after the last injections. Sera were heated at 56°C for 30 minutes and stored at -20°C. As preliminary *in vivo* tests failed to show anaemia developing in the recipients no absorption with murine red cells was done. Likewise, control serum from normal rabbits (NRS) was decanted at 56°C and not absorbed.

Treatment. 3 groups of mice received daily 6 times a week for 3 weeks 0.2 ml of either normal saline, normal rabbit serum (NRS), or rabbit anti mouse anti lymph node cell serum (ALS). Intraperitoneally. In addition, all animals received simultaneous daily injections of 0.5 ml 5 per cent sodium caseinate subcutaneously. In all animals leukocyte and differential counts were made 2-3 times weekly during the experiment. For this purpose blood was drawn from the retroorbital plexus in heparinized calibrated capillary tubes and countings were performed by standard methods. Animals dying spontaneously in the experimental period were excluded. The day after the last injection all surviving animals were killed by ether inhalation. Tissues were fixed in neutral formalin and paraffin embedded sections were stained with haematoxylin-eosin, methyl green pyronin, the PAS technique, alkaline Congo red and with thioflavin T. Amyloid was identified by its morphology and by its birefringence in Congo stained sections when viewed under crossed polars.

RESULTS

Effects on lymphoid tissues. In the two control groups receiving casein + saline and casein + NRS no reduction in the numbers of circulating lymphocytes was seen, the average during the whole experiment being between 4,000 and 5,000 per cmm blood. In the experimental group, however, receiving casein + ALS the lymphocyte counts rapidly decreased during the first two days until stabilizing around or below 1,000 per cmm blood during the rest of the experimental period (no attempt has been made to distinguish between large, medium-sized and small lymphocytes). The average numbers of circulating mononuclear cells for each group during the experimental period are graphically

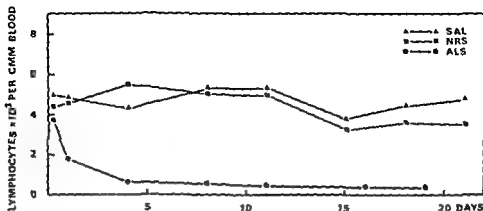


Fig 1

Mean numbers of circulating mononuclear white cells in three groups of casein treated C3H mice, simultaneously receiving intraperitoneal injections (daily) of either physiological saline (SAL), normal rabbit serum (NRS) or rabbit anti mouse anti lymph node cell antiserum (ALS)

outlined in Fig 1. After the first week a uniform transitory granulocytosis ranging from 10,000 to 25,000 per cmm blood was evident in about half the animals of each group.

Lymph nodes, spleens and thymus glands from the group receiving casein + ALS were not weighed, but judged by their appearance they were not reduced in size as compared with those of the controls. The lymph nodes from the two control groups treated with casein + saline and with casein + NRS, respectively, showed no histologic changes other than those usually seen developing after a protracted antigenic stimulation—a general hyperplasia and increased cellularity of germinal centres. In contrast the lymph nodes of the animals receiving casein + ALS showed a marked depletion of lymphocytes, the majority of cells being reticular cells and histiocytes. A rather constant feature was the accumulation in the lymph nodes of histiocytes with a cytoplasm distended by a large round, bright PAS-positive inclusion (Fig 2).

Histologically the spleens of the animals treated with casein + ALS presented a uniform though rather chaotic picture (Figs 3-5). The Malpighian corpuscles were markedly reduced in number and size due

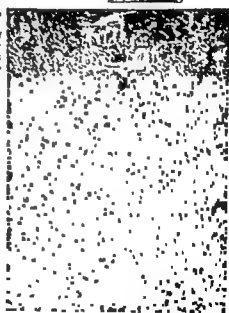
Figs 2-5

Fig 2 Lymph node from ALS treated mouse. Apart from a pronounced lymphoid depletion an accumulation of histiocytes with large bright PAS positive inclusions are seen PAS stain ($\times 350$).

Fig 3 Severe lymphoid depletion of spleen from ALS-treated mouse. Alkaline Congo red ($\times 35$).

Fig 4 Thymus and lymph node from ALS treated mouse. Note the marked lymphoid depletion of the lymph node in contrast to the intact thymic cortex. Alkaline Congo red ($\times 35$).

Fig 5 Spleen from ALS treated mouse. Around the depleted Malpighian corpuscle many plasma cells. Alkaline Congo red ($\times 140$).



to an excessive depletion of lymphoid cells. Surrounding the central artery was a loose connective tissue in which clusters of large pyroninophilic reticular cells were seen among which many plasmacytoid cells. In addition a small number of mature plasma cells. The splenic red pulp contained numerous histiocytes plasma cells and scattered groups of pyroninophilic reticular cells. Many mitoses were seen. Conversely the control spleens showed increased cellularity and large Malpighian corpuscles.

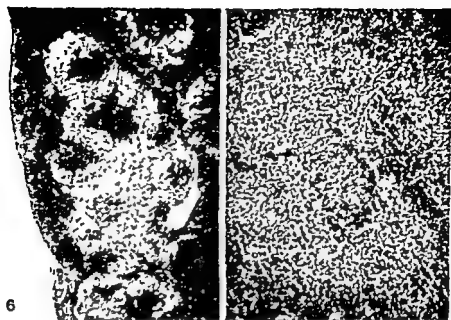
The thymus glands of the control groups showed no abnormalities. In animals receiving casein + AIS the thymic structure was in all cases intact. In a few animals a slight cortical depletion was noted (Fig. 4).

TABLE 1

Mean Numbers of Circulating Lymphoid Cells and Mean Degrees of Spleen Amyloidosis in 3 Groups of Mice Treated for 3 weeks with either Casein + Normal Saline, Casein + Normal Rabbit Serum (NRS) or Casein + Rabbit Anti Mouse Anti Lymph Node Cell Antiserum (ALS)

Treatment	Mean number of circulating mononuclear white cells during experiment (per cmm)	Number of animals developing amyloidosis Total	Mean degree of spleen amyloidosis
Casein + Saline	4707	9/9	++++
Casein + NRS	4529	10/10	++++
Casein + ALS	691	4/9	+

Effects on amyloid formation. As shown in Table 1 only 4 out of 9 surviving animals of the group receiving ALS developed amyloid as a result of the casein treatment (among 3 more animals of this group dying spontaneously no amyloid occurred). The amount of amyloid formed in these 4 mice was far less than that of the corresponding controls ranging from (+) to ++ as compared to an average of ++++ in the control groups. In these all mice developed splenic amyloid to about the same extent and their amyloid deposits were found uniformly distributed as fairly regular perifollicular rings throughout the spleens (Fig. 6). However the animals receiving casein + ALS if amyloidotic showed a distribution of the amyloid quite different from that of the two control groups. In some cases only 2 or 3 follicles per spleen section were involved in the amyloid formation. Apart from this the sites of amyloid deposition within the spleens were similar to those of the controls, the amyloid tending to stick to the perifollicular regions. If a Malpighian corpuscle was almost or completely absent the amyloid would precipitate in the periphery of its remnants or around the central artery (Fig. 7). In the two control groups small amounts of amyloid could be recognized in close rela-



Figs 6-7

- Fig 6 Spleen amyloidosis in NRS treated control, grade + + + Haematoxylin eosin ($\times 35$)
- Fig 7 Small amounts of amyloid surrounding remnants of a Malpighian corpuscle in an ALS-treated mouse Grade + Alkaline Congo red ($\times 140$)

tionship to the Kupffer cells lining the sinusoids of the liver and, occasionally, in the kidneys. In the animals of the ALS-group no hepatic or renal amyloid could be found.

DISCUSSION

In the present experiment, apart from causing severe lymphoid depletion, injections of ALS simultaneous with casein seemed to prevent the formation of amyloid in 5 out of 9 mice and markedly to reduce the degree and severity of amyloidosis in the remaining 4 mice. These 4 animals also showed a different distribution of the small amounts of amyloid substance present within the spleens.

The most extensive studies on the various properties of antilymphocytic sera in mice hitherto reported were those of Gray *et al* (1966) and Vonaco *et al* (1966). They found that both the cytotoxic and the leukoagglutinating activities were reduced by prior absorption with spleen and lymph node cells but not with liver or kidney cells. In the same way the *in vivo* lymphopenia effect could be removed. Cytotoxicity was shown to be complement dependent. They found ALS to be species—but not strain-specific. Their findings further suggested that the antilymphocytic properties of ALS were not due to antibody pre-

sent in the ALS directed against mouse gamma globulins. They found lymphopenia maintained during several weeks of treatment and for one week after, other peripheral leukocytes actually being increased. The treatment with ALS was well tolerated, no weight loss or renal damage occurred. Mice receiving ALS showed an elevated level of circulating cortico-steroid hormones as compared to NRS-treated controls. This raise was probably a response to cell destruction by ALS rather than being responsible for the lymphopenia. The histologic effects were a marked depletion of lymphoid cells while epithelial elements were preserved, complete repopulation did not occur until 6 weeks after the termination of serum therapy. ALS was found to markedly suppress both first- and second-set skin homograft rejection. Further, a significant inhibition of the primary humoral antibody response was observed, while no such inhibition occurred in the secondary response.

Waksman *et al* (1961) found ALS in guinea pigs exerting a suppressive effect on each type of delayed hypersensitivity reaction presumed to be mediated by cells, the tuberculin and contact reactions were completely abolished. Experimental, allergic encephalomyelitis showed a decrease in both incidence and severity and a marked delay in onset. There was a similar delay in the rejection of first-set skin homografts. However, the ALS had no effect on passive cutaneous anaphylaxis and the reversed passive Arthus' reaction, both mediated by circulating antibody. Nagaya & Steker (1965, 1966) observed only animals treated with antiserum to thymus showing depletion of thymocytes whereas thymus glands in animals receiving antiserum to lymph node cells remained intact. This is to some degree in keeping with the findings in the present experiment where thymus glands of animals treated with ALS were largely unaffected in contrast to the marked lymphopenia observed in spleens and lymph nodes. However the ALS-treated mice of Gray *et al* (1966) showed severe cortical depletion of the thymus. Nagaya & Steker found significant prolongation of skin graft survival and depression of delayed skin reaction to bovine serum albumin in ALS-treated rats. In keeping with this observation are the findings of Russe & Crowle (1965) that mice treated with anti-thymocyte serum failed to develop hypersensitivity to antigens employed early in the experiment, or they developed the hypersensitivity with slower than normal tempo.

In the present experiment and in the experiments of Sacks *et al* (1964), Monaco *et al* (1965), Nagaya & Steker (1965, 1966) and Gray *et al* (1966) it was possible to maintain a persistent lymphopenia for more than 3 weeks. In all the investigations cited immunologic depression was evident during the whole period of induced lymphopenia. In the investigations of Woodruff & Anderson (1964) some animals seemingly developed long-term or permanent tolerance to skin homografts after the termination of ALS treatment.

The above mentioned suppressive effects on cellular or delayed

immune mechanisms of an immunologically induced lymphopenia correspond well with the well known immune-suppressive effects of less specific lymphopenia inducing methods, in particular the use of x irradiation and cortisone. In view of the similarities between the inhibitive action of both cortisone when applied in the early phase (*Christensen 1961*), and ALS on the development of experimental amyloidosis and the general immune suppressive action of both cortisone and ALS it seems justified to assume that the amyloid inhibiting effect rely primarily on an inhibition of the immune process involved in the pathogenesis of experimental amyloidosis. Evidence that this immune process is in some way akin to cellular or delayed immune reactions has been collected (*Rantow 1966*). In keeping with this assumption are the several indications cited above that antilymphocytic serum mainly acts in suppressing cellular immune responses. ALS inhibition of amyloid formation may thus be considered caused by a selective suppression of immune mechanisms akin to the cellular or delayed type mediated by lymphoid cells where as cortisone inhibition is caused by a less specific, general immune suppression. Like cortisone, ALS will probably prove to exert an enhancing effect on amyloid formation if applied during the second phase of amyloid induction (*Teitum 1962*).

However, it must be borne in mind that the ALS can not be said to be truly an "antilymphocytic serum". Rather is it an antiserum against a crude lymph node cell suspension containing cellular antigens representing not only small lymphocytes but also various "reticular" cells. The possible role of these reticular cells in amyloid formation has earlier been stressed by *Teitum (1956 1961)*. On the other hand the relationship between these cells and small lymphocytes and their potential interchange under conditions of antigenic stimulation (*Gowans 1962*) are by no means clear which adds to the difficulties in interpreting results obtained with anti lymph node cell antiserum. Similar experiments employing a "pure" antilymphocytic antiserum prepared against thoracic duct small lymphocytes will probably provide some information regarding the cell type or types involved in amyloid formation under experimental conditions.

SUMMARY

In continuation of earlier research on enhancing and inhibiting factors in experimental amyloidosis the effects of antilymphocytic serum (ALS) on the development of amyloidosis in casein treated mice have been investigated. It appeared that small incus treatment with casein and ALS prevented amyloid formation in the majority of mice and greatly reduced its severity among the remainder.

The results are discussed in view of *Teitum's* pathogenetic theory and some bearings upon the nature of the immune reaction involved in experimental amyloidosis are briefly outlined.

cysteine hydrochloride, 1 per cent glucose and 5 per cent human ascitic fluid (cultivation was carried out in 500 ml round, flat-bottomed flasks filled up to the neck with medium and properly plugged). The bacteria were harvested by centrifugation after incubation at 37° C. for 2 or 3 days.

Tests for physiological properties. All tests for physiological properties were made in the enriched nutrient broth medium but the ascitic fluid content was reduced to 2 per cent. Cultivation was carried out in screw cap bottles (15 ml) filled to the top with medium.

Production of indol and hydrogen sulphide was tested with Kovac's reagent and lead acetate test strips, respectively.

Gelatine liquefaction was tested with 12 per cent gelatine added to the fluid medium.

Reduction of nitrate was examined in the enriched broth medium supplemented with 0.1 per cent potassium nitrate, as described in (2).

All tests were performed after 2 or 3 days incubation at 37° C.

The fermentation studies were carried out by replacing glucose by a series of other carbohydrates (cf. results), which were added separately to the sterile solutions to give a final concentration of 1 per cent. The pH of the fluid media was adjusted to 7.4. After 4 days incubation at 37° C., the pH was determined by a Beckman pH meter.

Serological Methods

Immunization. Rabbit antisera were produced by intravenous injections of washed microbes using the dosage scheme described by Oeding (9). The antisera against the washed bacteria contained precipitating antibodies against the ascitic component of the culture medium and had to be absorbed with ascitic fluid before use. The absorptions were carried out at 37° C. for 2 hours and the specimens were then left in the refrigerator overnight before centrifugation. One volume of ascitic fluid was used for absorption of 50 volumes of antiserum.

Ring test precipitation and complement fixation were performed as described in (5). Antigenic material was obtained by extracting whole microbes overnight at 37° C. with 0.02 M phosphate buffer of pH 7.4.

Agglutination was carried out in tubes. Two drops of a saline suspension of trypsin digested microbes (turbidity corresponding to McFarland's scale no. 10) were added to 0.5 ml of serum in dilutions. The agglutination was read with the naked eye after incubation at 37° C. in the thermostat for 20 hours.

Indirect haemagglutination. Washed sheep erythrocytes were sensitized as described by Morse (7). Extract of 200 mg bacteria (wet weight) was used for sensitization of 0.1 ml packed sheep cells. The haemagglutination tests were performed as described by Oeding *et al.* (10) but the haemagglutination was recorded by reading the patterns (11).

RESULTS

Morphology and Some Physiological Characteristics

The morphology and the physiological properties of the three *L. buccalis* strains included in the present study corresponded to earlier descriptions of this species. The atypical filiforms differed from *L. buccalis* in their morphology, and in their capacity to produce gas and indol in fluid media (Table I). Morphologically the atypical filiforms appeared as straight or slightly curved segmented bacilli or filaments with rounded or blunt ends. The thickness and the length of the bacterial cells varied from strain to strain, and also somewhat with the cultural conditions. Long filaments were particularly seen in old cultures in fluid media. Young cells of the type I strains (cf. Table I) were relatively small and slender, about 1 μ thick and 10 μ long. The type II strains were more pleomorphic and varied within a greater

range from one strain to another. Both types were frequently arranged in chains. Central or subterminal swellings which did not take up a spore stain were encountered most frequently in type II strains.

TABLE 1

Morphological and Physiological Differences between the Atypical Filiforms and L. buccalis

Strains		Morphology		Production of	
Type	Number	Bacillary	Filiform	Gas	Inol
Atypical filiforms					
Type I	15	15	0	15	15
Type II	12	12	0	12	12
<i>L. buccalis</i>	3	0	3	0	0

Type I Colonies filiform and adherent

Type II Colonies varying in adherence

The atypical filiforms showed the same liability to Gram staining as *L. buccalis*. Exponentially growing cultures incubated overnight or longer contained both Gram positive and Gram negative cells, the latter frequently with Gram positive granules. Branching was never seen. None of the strains were motile.

Colonies on blood agar of type I strains were colourless and had a tiny central eminence from which interlacing ridges radiated to filamentous edges, giving the whole colony an extremely rough and flat appearance. The type I strains were all adherent to the solid medium but gelatinous and easily emulsified. The colonies of the type II strains varied with the different strains from the filamentous appearance of the type I strains to the smooth and shiny convoluted brain shaped or Medusa head appearance of *L. buccalis* colonies. They were not adherent to the solid medium.

In fluid media the atypical filiforms grew with granular or flocculent sediment which was sometimes adherent to the side or bottom of the flasks.

TABLE 2

Fermentation Patterns of Atypical Filiforms and L. buccalis

Strains		No. of strains fermenting					
Type	Number	Lactose	Trehalose	Sucrose	Raffinose	Xylose	Starch
Atypical filamentous							
Type I	5	5	5	3	5	4	5
Type II	7	7	7	7	7	5	7
<i>L. buccalis</i>	3	1	2	3	0	1	2

All strains fermented glucose, lactose, maltose, sucrose and mannose. None of the strains fermented mannitol, dulcitol, salicin, rhamnose, sorbitol or glycerin.

a weak serological cross-reactivity or none at all between the filamentous organisms and *L. buccalis*. Further studies are needed to clarify the taxonomical position of the atypical filamentous bacterium, although much is in favour of classifying it as a new *Leptotrichia* species, the name of which should be *Leptotrichia aerogenes*.

It is worth emphasizing the finding of antibodies to *L. buccalis* in non-immunized rabbits. Antibodies to *L. buccalis* have also been demonstrated in normal human sera (6).

SUMMARY

Some morphological, physiological and serological data concerning an anaerobic oral filamentous organism are described. The bacterium has several properties in common with *Leptotrichia buccalis*, but differs from that organism morphologically and serologically, and in its capacity to produce gas and indol in fluid media.

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PRESENCE OF A VASO ACTIVE FACTOR IN A NEPHRITOGENIC GROUP A, TYPE 12 STREPTOCOCCAL STRAIN

By

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Studies in animals concerning hypertension in experimental glomerulonephritis induced by streptococci have been reported by *Reed & Matheson* (1954a, b), *Matheson & Reed* (1954) and *Miyamoto et al* (1960). The firstmentioned authors were able to produce nephritis and hypertension in rabbits both by infection with nephritogenic group A, type 12 streptococci and by injection of culture filtrates from these strains. From these filtrates they precipitated with ammonium sulfate a peptide which had nephritogenic and hypertensive properties. The kidney injury involved particularly the tubulus apparatus but slight or no glomerular changes were noted. *Miyamoto et al* (1960) reported successful results in inducing typical diffuse glomerulonephritis and hypertension in rabbits infected with type 12 streptococci employing Okabayashi's intranasal sinus window method. While the triad hematuria, hypertension and albuminuria appeared after 18 to 21 days in *Reed & Matheson's* experiments *Miyamoto et al* (1960) noticed hematuria, oedema and hypertension after a latent period of 6 to 7 days. In all these investigations the ear capsule method was used for the registration of blood pressure. The same method was also employed by *Holm et al* (1966) in studies concerning experimental streptococcal glomerulonephritis in rabbits. Besides a typical glomerulonephritis the latter authors were also able to produce increased blood pressure in the animals which started within a few minutes after the injection of streptococcal cell materials from a nephritogenic strain of type 12 streptococci. The possibility of the presence of a hypertensive factor in

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nephritogenic streptococci was assumed. The aim of the present investigation was to analyse an "autolysate" preparation from a nephritogenic streptococcal strain for the presence of a vaso-active factor and to characterize this factor from a chemical point of view.

MATERIAL AND METHODS

Streptococcal autolysate. A nephritogenic streptococcal strain (Group A, type 12 strain P 13846) was grown under stabilized pH conditions in an antigen free medium prepared according to the description of *Holm & Falsen* (1966). The streptococcal cells were harvested, treated and subjected to autolysis as described by *Holm et al* (1966). The autolysate preparation after 10 times concentration on a Buchi Rotavapor R apparatus at 33° C. was used in the gel filtration experiments.

Gel filtration of concentrated streptococcal autolysate. Gel filtrations were performed on 1 × 180 cm columns using Sephadex G-25 fine bead polymerized (AB Pharmacia Uppsala, Sweden). A volume of 25 ml of the concentrated autolysate was introduced on the top of the column and eluted with newly distilled water or M/1000 phosphate buffer (pH 7.4) at room temperature. The flow rate through the column was adjusted to 20 ml/hour and the material collected in 5 ml volumes by means of a siphon-controlled fraction collector (L&B, Stockholm, Sweden). The optical density of each 5 ml portion was measured at 260 mμ in a Beckman DU spectrometer.

Ultraviolet absorption spectrum. The gel filtration eluates as well as reference substances were scanned on a Unicam SP 800 apparatus.

Infra red spectrophotometry. Infra red absorption patterns were obtained in a Beckman IR 7 spectrophotometer. Materials to be analysed were dried over P₂O₅ mixed with KBr (1:400) and KBr discs prepared. The IR patterns obtained were interpreted according to *Cross* (1960).

Animals. Rabbits weighing between 1.6 and 4.5 kg were used in the animal experiments. They were sacrificed between 2 and 3 weeks after the beginning of the individual experiments.

Blood pressure readings. The blood pressure in rabbits was measured by the ear capsule method of *Grant & Rotshild* (1934). In some experiments the intra arterial blood pressure was also recorded using a calibrated Honeywell 1508 viscorder connected via a catheter to the left femoral artery of the rabbit.

Antiserum. Antiserum against streptococcal material was obtained from a sheep which had been hyperimmunized with intra- and extracellular material from dif-

ferent strains of streptococci. The antiserum was precipitated with 50% saturated ammonium sulphate, washed with distilled water, and then dialysed against distilled water.

The tissues were stained with hematoxylin & Giesson and examined for histopathological changes.

EXPERIMENTS AND RESULTS

Gel Filtration of Concentrated Autolysate

The results of a typical fractionation experiment of the concentrated streptococcal autolysate on Sephadex G-25 employing phosphate buffer as eluent are illustrated in Fig. 1. No differences in results were obtained when distilled water was used instead of phosphate buffer as effluent. Six main peaks were seen when the eluted material was analysed at 260 mμ and the effluent was pooled into six fractions as indicated in the figure. The fractions were concentrated to one-tenth of the fraction volume on a Buchi Rotavapor R at 33° C. and stored at -20° C. All of the antigenic material was recovered in fraction 1.

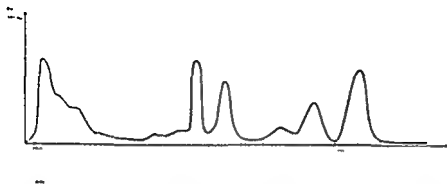


Fig 1

A typical elution diagram of concentrated streptococcal autolysate fractionated on a 1×180 cm Sephadex C 25 column. The shaded part of the curve represents fraction VI containing the material with vaso-active capacity.

(eluted with the void volume of the column) according to diffusion in gel analysis with the antistreptococcal serum. Fraction VI was collected after the elution of 160 ml corresponding to 4.2 times the void volume of the column. Subsequent filtration of reference substances (e.g. adenine Fluka) through the same column permitted a rough estimation of a molecular weight 120-150 for materials eluted with fraction VI.

The ultraviolet absorption curve of fraction VI showed a distinct maximum at 260 $m\mu$ and minima were found at 227 $m\mu$ and 287 $m\mu$.

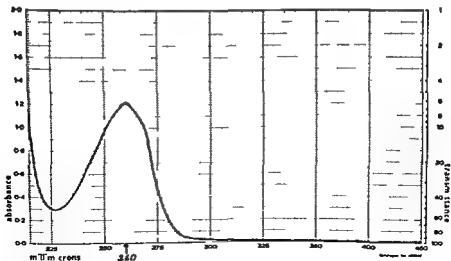


Fig 2

Figure illustrating the UV absorption curve of fraction VI. The curve was obtained by scanning the material in a Ducon SP 800.

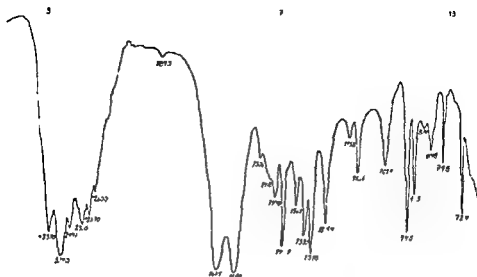


Fig 3a

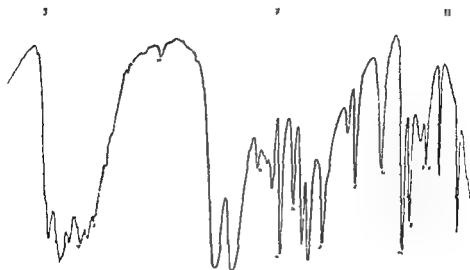


Fig 3b

Fig 3a and b

Infra red spectrum of a dried material from fraction VI and b adenine (Fluka). The numbers above the curves refer to the wavelength in microns. The numbers below the peaks refer to frequency in cm^{-1} , recorded on a Beckman IR 7 model.

From the UV curve illustrated in Fig 2 it may also be seen that the curve was not symmetrical but showed a shoulder phenomenon at 268 $\text{m}\mu$.

Infra red spectrophotometry of dried material from fraction VI (eluted with distilled water) showed several distinct absorption maxima as may be seen in Fig 3a. Although the presence of impurities

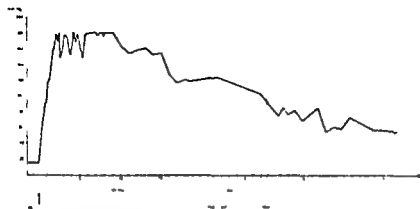


Fig. 4

Diagram of blood pressure in rabbits injected with material from fraction VI. The registrations were made with the ear capsule method.

in the material could not be excluded the principal structure closely resembled that of adenine (Fig. 3b).

Vaso-Activity of Concentrated Autolysate and Fractions thereof

Investigations concerning the vaso-activity of concentrated autolysate and the six fractions obtained at gel filtration were performed in rabbits. An aliquot of 0.2 ml of each preparation was injected intravenously into each of three rabbits and the blood pressure recorded by the ear capsule method. Simultaneously observations concerning changes in the ear blood vessels were made. After the injection of concentrated autolysate an elevation of the blood pressure was registered up to 180 mm Hg within 2-3 min. A slow decrease was then noticed during the following hours but the blood pressure was still high two weeks after the injection (60 mm Hg). The sudden elevation of the blood pressure was accompanied by a pronounced dilation of the ear vessels. This dilatation slowly decreased parallel to the blood pressure during the following weeks.

After injection of fractions I-III no blood pressure increase was registered while fractions IV and V induced a slight elevation of the blood pressure not exceeding 45 mm Hg. Only a slight dilatation of the ear vessels was observed in relation to injection of fractions IV and V.

Fig. 4 illustrates a typical blood pressure curve from one of the rabbits injected with fraction VI. As may be seen the maximal blood pressure (180 mm Hg) was registered 2-3 min. after the injection. In accordance with the results obtained after injection of concentrated autolysate the blood pressure fluctuated within a wide range during the first 3 min. A slow decrease was then noted during the following hours but the blood pressure was still high in all three rabbits (50-60

mm Hg) two weeks after the injection. A pronounced dilatation of the blood vessels was also observed after injection of fraction VI. The dilatation was maximal 2-3 mm after the injection and then slowly subsided parallel to the decrease in blood pressure.

An additional two rabbits were injected intravenously with 0.2 ml adenine in saline (Fluka 0.1 mg/ml). No dilatation of the ear arteries was observed and the blood pressure as measured by the ear capsule method remained within normal limits throughout the two week experimental period.

The Effect of Fraction VI on Intra Arterial Blood Pressure

To investigate the observed vascular reactions the following experiments were performed. Two rabbits were anesthetized with Nembutal and the left femoral artery was dissected free. The artery was then connected with a Honeywell visicorder via catheter and the initial systolic blood pressure (135-150 mm Hg) was recorded. Simultaneously, blood pressure readings were made with the ear capsule method. After intravenous injection of 0.2 ml of material from fraction VI no elevation of the blood pressure could be registered in the femoral artery during the following two hours while with the ear capsule method an increase from the initial 20-25 mm Hg up to 140 mm Hg was noted within a few minutes followed by a slight decrease during the next two hours.

Histopathological Examinations

Kidney sections from all rabbits injected with material from fraction VI were analysed histopathologically. No signs of kidney lesions could be detected.

DISCUSSION

In reports concerning experimentally induced streptococcal nephritis Reed & Matheson (1954a, b), Matheson & Reed (1959), Miyamoto *et al* (1960), Holm *et al* (1966) have reported increased blood pressure in rabbits as registered by the ear capsule method in addition to kidney lesions. The hypertension was however obtained at different times. Reed & Matheson (1954a, b) reported a gradual development of the hypertension similar to that noted with glomerulonephritis in humans. Holm *et al* (1966) observed hypertension within a few minutes after the injection of streptococcal material. It may be assumed that two independent factors exist in streptococci, one being primarily nephrotoxic and the other being responsible for the immediate elevation of blood pressure. As earlier reported by Jonsson & Holm (1964) similar effects could not be produced by a non-nephritogenic type 7 streptococcal strain.

The present results indicate that a "hypertensive" factor could be

separated by gel filtration from the autolysate preparation (fraction VI). That this fraction was free from precipitinogens and lacked nephrotoxic capacity was also shown. Using reference substances like adenine the molecular weight of materials eluted with fraction VI was roughly estimated at 120-150.

Although the IR spectra for adenine and the material eluted with fraction VI were similar the presence of impurities in the latter material cannot be excluded. However, the amount of impurities should be very small as judged by the distinct peaks in the spectrum. The shoulder phenomenon at 268 $m\mu$ in the UV curve of material from fraction VI (not present in the UV curve of adenine) should then represent an impurity with an unusual high molar extinction equal to that of adenine and adenine derivatives. It must therefore be assumed that the material in fraction VI contains adenine and chemical compounds similar to adenine, or adenine and a hitherto unknown substance with an extremely high molar extinction, the latter suggestion being improbable. As pure adenine lacked a "hypertensive" effect in rabbits, as measured by the ear capsule method, it may be concluded that the "hypertensive" activity of fraction VI is most probably caused by a substance similar to although not identical with, adenine.

Intra arterial recordings of blood pressure in anesthetized rabbits simultaneous to blood pressure readings by means of the ear capsule method showed that a blood pressure increase as registered by the latter method did not correspond to an elevation of central blood pressure. From this experiment it may thus be concluded that fraction VI contained a substance with vaso-dilatative capacity simulating blood pressure increase noted with the ear capsule method but devoid of central hypertensive effect. Whether this effect in rabbits is only localized to ear arteries or may also involve other vessels *e.g.* those in the kidney, cannot be stated at present. If the smaller kidney arteries or even the glomerular capillaries should react in a similar way this might offer a new explanation for the location of streptococcal nephritotoxic products in the kidneys in the natural course of human glomerulonephritis.

SUMMARY

A substance with a low molecular weight was separated from the precipitinogens in an autolysate from a nephritogenic, type 12, streptococcal strain by gel filtration through Sephadex \square 25. Intravenous injection of the substance into rabbits resulted in a pronounced dilatation of the ear arteries and elevated blood pressure which were observed with the ear capsule method. However, no elevation of the central blood pressure (A femoralis) was noted. The streptococcal substance responsible for the vaso activity is most probably an adenine-derivate and was found to lack nephrotoxic capacity in rat.

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BODY TEMPERATURE, ANTIBODY FORMATION AND INFLAMMATORY RESPONSE

By

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In 1897 *Widal & Sicard* (1) showed that poikilothermic animals were good antibody producers at high temperatures, at for instance 37° C. Antibody production was reduced at temperatures between 21° C and 33° C and completely abolished at 12° C. Similar results were achieved by *Sybelin* (2) in 1935.

In a preliminary report *Wanler* (3) showed that hibernating hedgehogs with a body temperature of 6° C did not produce antibodies and showed no local inflammatory reaction whereas they gave an active response when they were awake and warm with a body temperature of 33° C.

The ability to produce antibodies has thus been quite thoroughly studied in experimental animals, but few have paid any attention to the influence of body temperature on the local inflammatory response. This problem has been studied during hypothermia under anaesthesia in a series of papers by *Sranes* (4). He found that warm-blooded animals showed a marked reduction in local inflammation when the body temperature was reduced to 20° C-22° C.

In this communication we have repeated the experiments from 1962. As experimental animals we have used hedgehogs and also fish: cod and gold-fish. We wished to avoid the nonphysiological condition which occur in warm blooded animals during hypothermia under anaesthesia. In poikilothermic animals such as fish for instance, it is easy to change the temperature of the surrounding medium in order to produce variations in body temperature, and the condition of the experimental animal should be physiological, at least more so than during hypothermia and anaesthesia in warm-blooded animals. Hedgehogs are particularly easy to study from this point of view. In the summer season they have a body temperature of 32°-33° C. If they are placed in a refrigerator at a temperature of 2-6° C they go to sleep and the body temperature drops to around 6° C. They can stay in hibernation for 3-4 months, but wake up if they are taken out of the refrigerator and placed in a room with a temperature of 18°-20° C. The body temperature then goes up to values

normal in the non-hibernating state. As a rule the animals will stay awake in winter if they are kept at ordinary room temperature and are fed regularly. They may however go to sleep for shorter or longer periods and stay in a "sub-hibernating state" with a body temperature of between 18°-26° C. As a rule it is easy to get adequate controls.

MATERIAL AND METHODS

Hedgehogs Altogether 18 hedgehogs were used, 9 non hibernating and 9 in the hibernating state. In order to procedure hibernation the animals were placed in a refrigerator, and the body temperature of the animals was checked at regular intervals.

Inflammatory response 2 animals in each group were injected with 0.2 cc turpentine intraperitoneally. The animals were killed after 24 hours. The local inflammatory response was studied macroscopically and in smears.

7 animals in each group were injected subcutaneously with 0.5 cc of an emulsion of silica (2 per cent emulsion in normal saline, particle size 1 micron). In 5 animals in each group the injected area was found at the end of the experiment (3 months-4 1/2 months) and adequate histological examination of the material could be carried out.

Antibody formation Two of the experimental animals in each group in the last experiment were given parathyphoid B vaccine and washed sheep red cells intraperitoneally. The animals were given 4-6 intraperitoneal injections in the last month of the experiment. The hibernating animals had then been sleeping for 3 months. The last injection was given one week before the termination of the experiment and blood was taken for agglutination and complement fixation tests.

Fish The local inflammatory response following the intramuscular injection of 2 per cent silica in 0.15 per cent saline was studied in cod and gold fish. Only pilot experiments were performed as one animal in each group was examined. The cod's temperatures were 18° C for the warm and 7° C for the cold. In the gold fish the temperatures were 22.5° C and 8° C respectively. The warm cod did not thrive and died after 7 days whereas its cold counterpart was active and healthy. In the same way the cold gold fish did not stand the low temperature well and died after 7 days whereas the warm showed normal activity. Apparently these animals are too sensitive to great variations in temperature, and we have been too radical in our experimental procedure.

TABLE 1
Antibody Formation in Hedgehog

Animal No	Sheep cells		Animal No	Para B	
	aggl	compl fix		O aggl	II-aggl
1	80	80	6	572	572
2	80	80	7	64	572
3	0*	0*	8	32	0†
4	0	0	9	not tested	

No. 1, 2, 6 and 7 non hibernating

No. 3, 4, 8 and 9 hibernating

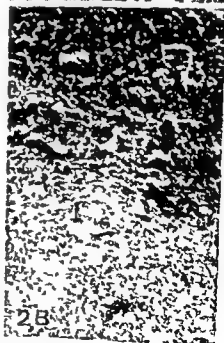
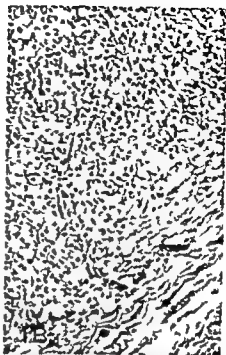
* Highest concentration tested 1/4

† Highest concentration tested 1/10

FIGS 1 & 2

Figs 1 A and B Silica nodule in warm non hibernating hedgehog. Solid granules with macrophages. H.E. A $\times 35$ B $\times 140$

Figs 2 A and B Silica nodule from a hedgehog in the "sub hibernating state". Peripherical infiltration of lymphocytes and macrophages. Central area few cells. H.E. A $\times 35$ B $\times 140$



Antibody Formation in Hedgehogs

Our results are summarized in Table 1. The difference between the two groups is evident. Active antibody formation in the warm non hibernating animals no or insignificant activity in the cold hibernating group. Reading of the pratyphoid O agglutination was difficult. Apparently the animals showed some normal O agglutins. Further examination and evaluation of this doubtful finding is necessary.

Local Inflammation in Hedgehogs

Our first experiment with acute inflammation after turpentine injection showed no definite difference between the cold and the warm hedgehogs. There were possibly not so many polymorphnuclears in the smears from the sleeping hedgehog as compared to the warm animal but the difference was insignificant. However the hibernating animal was apparently not asleep at the end of the experiment. A new trial was therefore carried out with temperature control. We found that during the experiment the temperature of the hibernating animal increased to above 30°C and a adequate control could therefore not be run. This inflammatory stimulus is too great because it interferes with the hibernation. For this reason we selected silica emulsion for our next experiments.

TABLE 2
Local Silica Inflammation in Hedgehogs

Animal No	Granuloma formation	Condition of animal
1	Solid granuloma with macrophages	Awake Temp 32°C
2	Peripheral cellular reaction with macrophages. Centre Few cells	Partly sleeping Temp $18^{\circ}20^{\circ}\text{C}$
4	No cellular reaction	Hibernating Temp $6^{\circ}8^{\circ}\text{C}$

D duration of experiment 5 months

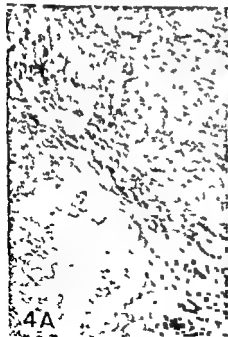
Silica Granulomas in Hedgehogs

Our results are summarized in Table 2. We got three types of reaction. The non hibernating animal (No 1) showed a solid granuloma with macrophages from the periphery to the centre (Figs. 1 A and B).

Figs. 3 & 4

Figs. 3 & 4 and B Silica nodule from a hibernating animal No cellular reaction III
A $\times 35$ B $\times 140$

Fig. 4 A Silica nodule from a hibernating animal No cellular reaction III $\times 150$
B Same field in polarized light showing the silica granules within the nodule



RESULTS

Antibody Formation in Hedgehogs

Our results are summarized in Table 1. The difference between the two groups is evident: Active antibody formation in the warm non-hibernating animals, no or insignificant activity in the cold hibernating group. Reading of the paratyphoid O-agglutination was difficult. Apparently the animals showed some normal O-agglutins. Further examination and evaluation of this doubtful finding is necessary.

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Duration of experiment 3 months

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Figs. 3 & 4

Figs. 3 & 4 and B Silica nodule from a hibernating animal. No cellular reaction. H.E. A × 35 B × 140

Fig. 4 A Silica nodule from a hibernating animal. No cellular reaction. H.E. × 150
B Same field in polarized light showing the silica granules within the nodule

One animal (No. 2) was partly awake and partly in a sub hibernating state with varying body temperatures below the normal for non hibernating animals. It showed a granuloma with macrophages and lymphocytes at the periphery and few cells in the central area of the nodule (Figs 2 A and B). Temperature control was only carried out in the last part of the experimental period and the animal probably had a "normal" temperature in the first part.

The hibernating animal (No. 4) showed a completely different histological picture. The silica remained in a definite nodule with hardly any cellular reaction (Figs 3 A and B). The experiments were repeated with similar results. This time no animal went into any kind of sub hibernating state. Silica was easily demonstrated in the nodules in polarized light. The nodules with the double refracting granulomas were clearly demonstrated in the hibernating animals and showed no infiltration of macrophages. Only a few fibroblasts were seen in the periphery surrounding the nodules as a thin capsule (Figs 4 A and B).

Silica Granuloma in Fish

The warm gold fish and the warm cod showed a definite cellular reaction with infiltration of lymphocytes and macrophages and proliferation of a few fibroblasts (Figs 5 A and B, Figs 7 A and B). The accumulation of doubly refracting silica granules within the inflamed area is nicely demonstrated. The cold animals on the other hand revealed only minor degenerative changes and no inflammation. The silica granules are seen in the non inflamed areas (Figs 6 A and B, Figs 8 A and B).

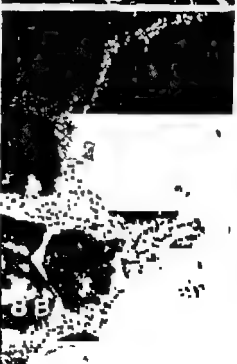
DISCUSSION

It is thus evident that formation of antibodies can not be demonstrated in the hibernating state. Apparently the reticulo-endothelial system is also sleeping but several questions have to be answered. What happens to the antigen during hibernation? Does it come into contact with the antibody producing organs? Is it possible that such contact in the hibernating animal may initiate a more rapid production of antibody when the animal wakes up? However these animals do not thrive and breed in captivity and it is not easy to get enough animals for experimental purposes.

Further it is evident that the low body temperature in the hibernating animals and in the gold fish and the cold cod does not permit a com-

Figs 5-6

F
F



SUMMARY

- 1 Hibernating hedgehogs with a body temperature of 5° C show no production of antibodies as contrasted to non hibernating animals with a body temperature of 32° C-33° C
- 2 Hibernating hedgehogs and cold eel and gold fish show no local inflammatory response after injection of silicemulsion. The reaction of the warm controls was marked
- 3 The influence of the body temperature upon the activity of the reticuloendothelial system the vaso active enzymes and capillary permeability is discussed shortly

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STUDIES ON ANTIGEN PREPARATIONS FROM *STAPHYLOCOCCUS AUREUS*

3. On the Homogeneity and Structure of Protein A

By

ANNE GROV

Received 23 ix 66

The *Staph. aureus* "group antigen" protein A (antigen A), which produces a precipitation line on agar gel diffusion against all normal human sera, has been shown to be of protein nature (6, 11, 12)

Serologically, our protein A preparation shows two precipitation lines against rabbit immune serum and the ability to sensitize tanned sheep erythrocytes. Chromatography and electrophoresis revealed no sign of heterogeneity (6, 15). The results of absorption experiments and chemical modifications of functional groups (4) strongly supported our suggestion that the different serological activities are due to separate molecular entities of the preparation.

The present paper deals with further investigations on the homogeneity of the protein A preparation, and the molecular size and shape of entities giving rise to the serological activities.

MATERIALS AND METHODS

Antigen preparations. The protein A preparation used was that described in (6). In addition a cell water extract was prepared according to *Edqvist & Sjoquist* (12). After precipitation with acid the supernatant was neutralized and subjected to serological tests.

Reagents. 2,4-dinitro 1-fluorobenzene (DNFB) was obtained from Merck, carboxy-peptidase-A (toluene water suspension) from Sigma Chemical Company, crystalline

Figure 1. Electrophoretogram of protein A preparation. The gel shows two distinct bands of protein A, indicating heterogeneity. The bands are labeled 1 and 2, corresponding to the two precipitation lines mentioned in the text.

acrylamide (10% solution) from Serva. Buffers of various pH values, all with ionic strength of 0.1, were employed and the time for each run was 3 hrs.

Peviskon bio E electrophoresis was carried out as described in (2) using Peviskon G-870 and a plexiglass tray of 27 × 15 × 0.7 cm. A constant voltage of 300 V was

The ultracentrifugation experiment was carried out at The Department of Biochemistry University of Bergen for which I am very much indebted to Dr Karen Helle.

supplied for 20 hrs giving a current of about 40 mA. Pieces of paper and Peckon one cm in width were cut off and extracted with distilled water. The extracts were then tested for serological activity.

Ultracentrifugation. Sedimentation velocity approach to equilibrium and diffusion were studied by means of the Spinco Model L analytical ultracentrifuge. A capillary type synthetic boundary cell was used in the diffusion experiment and for the approach to equilibrium run a double sector cell was used. All the ultracentrifugation experiments were performed at 20°C. The rotor speeds were 59780 RPM in the sedimentation velocity studies, 42040 RPM in the approach to equilibrium run and 10550 RPM in the diffusion study. Solvent density was determined by weighing measured volumes (Aga microvolumes) of solvent and water in weighed capped bottles at 20°C. The partial specific volume of the protein was calculated from its amino acid composition (16). In these experiments the concentration of protein A was 5 mg per ml in 0.05 M phosphate buffer pH 7.4.

Digestion with trypsin and carboxypeptidase was performed as described in (4). Free amino groups were determined by reaction with DNBS according to the description of Ghapure et al. (3). On determining free amino groups after tryptic digestion the same amount of enzyme was added to the blank as was used for digestion.

Quantitative determination of amino acids released by carboxypeptidase was carried out by letting protein A (5 mg) react with the enzyme (0.25 mg) for 24 hrs at 37°C (4). Thereafter the mixture was shaken for 2 hrs with a cation exchange resin (200 mg Amberlite MB 3 mixed bed). The supernatant protein solution was withdrawn and the resin was washed twice with water. The pH of the protein solution with washings added was adjusted and reprecipitated with carboxypeptidase performed. The combined washed ion exchange resin was then extracted for 2 hrs and washed twice by shaking with 5% NaCl. The extract with washings added was evaporated to dryness *in vacuo* over KOH pellets. The residue was dissolved in water and subjected to qualitative and quantitative paper chromatographic analyses as described in (5).

Treatment of immune serum and normal human serum with mercaptoethanol was performed by leaving a mixture of equal volumes of serum and 0.3 M 2-Mercaptoethanol (in saline) at room temperature for 24 hrs. In one series of experiments a 10 per cent molar excess of iodoacetamide was added to the reaction mixtures at 4°C for 1 hr, followed by dialysis against saline for 48 hrs. The treated sera were tested serologically. Sera mixed with saline instead of mercaptoethanol were always included and serum from a patient with infectious mononucleosis containing heterophile antibodies in high titre was included as control for the activity of mercaptoethanol.

Serological methods. Rabbit immune serum against the homologous strain C was produced as described by Oeding (14). The human serum used in this experiment was a pool of 10 normal human sera. The ring test and agar gel precipitation tests were carried out as described in (8) and (9) respectively and the indirect haemagglutination test essentially as described by Morse (13) using a 1:40,000 solution of tannic acid for tanning the sheep erythrocytes.

RESULTS

Serological examinations of the extracts from paper electrophoresis of our protein A preparation showed no separation of the activities. Neither was any separation achieved by Peckon block electrophoresis in citrate phosphate buffer at pH 4.1. On paper electrophoresis it was observed that all the serologically active material moved slowly towards the cathode at pH 8.0 and slowly towards the anode at pH 8.6 indicating that the isoelectric point of protein A is between these pH values. At more alkaline and more acid pH values the migration rate increased. Serologically active material was found in a rather broad band (5 to 6 cm) and some variation in the titres of the clones was observed.

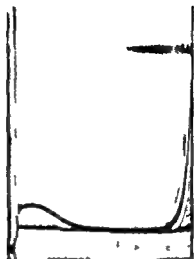


Fig. 1

Schlieren pattern of protein A (5 mg/ml in 0.05 M phosphate buffer pH 7.4) in the ultracentrifuge 161 mins after reaching 42 000 R.M.

During ultracentrifugation of protein A the Schlieren patterns showed a very slow migration. The observed single sedimentation boundary indicates homogeneity of the material (Fig. 1). From the experimental results the sedimentation coefficient ($s_{20,w}$) is estimated to 1.22 at 5 mg protein A per ml. A run with a concentration of protein A of 3.5 mg per ml gave an $s_{20,w}$ of 1.26 indicating higher value at infinite dilution. The diffusion coefficient ($D_{20,w}$) estimated from the diffusion experiment at 5 mg per ml of protein concentration is 14.966×10^{-7} cm²/sec and the partial specific volume (V_{app}) was calculated from the amino acid composition to give a value of 0.721 mg/ml. These data yield a value of 1.2 for the frictional ratio f/f_0 indicating a slightly asymmetric molecule (19).

The approach to equilibrium run according to Trautman (17) gives a slope of 3.298×10^3 and an average molecular weight (M_{app}) of 12 200. In the sedimentation study the apparent diffusion coefficient (D_{app}) increased with time (i.e. spreading of the boundary), demonstrating some degree of molecular heterogeneity (16). The increase of the diffusion coefficient was found to be 1.701×10^{-11} i.e. a small increase. The heterogeneity could not be detected by the approach to equilibrium centrifugation as the Trautman plot gave a straight line (17).

Based on a molecular weight of 12 000 the calculation of the number of amino acid residues gives a molecular composition as listed in Table 1. The total number of amino acid residues listed is 126 giving a molecular weight of 12 021.

TABLE 1

Amino Acid Composition, Integral Residues and Molecular Weight of Protein A

Amino acids	Amino acid residue (g/100 g dry protein)	Nearest integral number of amino acid residues for 12 000 g protein	Integral number of residues \times respective residue molecular weights
Lys	15.6	15	1920
Asp A	14.8	15	1725
Glu A	14.7	14	1806
Ser	5.9	9	783
Gly	12.3	26	1482
Ala	9.5	16	1136
Pro	12.3	15	1455
Val	6.5	8	792
Leu	7.4	8	904
H ₂ O*			18
Total	99.0	126	12021

* The molecular weight of water is added to correct for the mole of water on the terminal amino acids

The total amount of free amino groups present in our protein A preparation was estimated to 1.35 μ mole/mg, corresponding to 16 free amino groups per molecule (M_w 12,000)

Tryptic digestion increased the total amount of free amino groups to 2.59 μ mole/mg. Assuming a molecular weight of 12,000, the increase of free amino groups due to tryptic digestion corresponds to 15 amino groups per molecule

TABLE 2

Amino Acid Composition and Integral Residues of the Part of Protein A which was Digested by Carboxypeptidase

Amino acids	Amino acid residue (g/100 g dry protein)	Nearest integral number of amino acid residues for 12 000 g protein
Lys	1.3	1
Asp A	0.8	1
Glu A	0.9	1
Ser	0.7	1
Gly	0.5	1
Ala	1.2	2
Val	1.5	2
Leu	2.1	2
Total	9.0	11

After two consecutive digestions by carboxypeptidase no further release of amino acids was obtained. The quantities of amino acids released are listed in Table 2. In all, only about 9 per cent of the material was digested by this enzyme. The molar proportions of the

released amino acids. Isoleucine, aspartic acid, glutamic acid, serine, glycine, alanine, valine, and leucine were 1.07, 0.7, 0.8, 0.9, 1.7, 1.6, 1.8 respectively. In the same order the nearest integral number of amino acid residues should be 1, 1, 1, 1, 1, 2, 2, 2 based on a molecular weight of 12,000.

Whereas protein A gives a characteristic precipitation line on agar diffusion with all normal human sera, serum from a patient with agammaglobulinemia gave no line. This indicates that the normal serum component is a γ globulin. Owing to scarcity of the patient's serum, no other serological tests were performed. In the serological reactions with protein A and sera treated with mercaptoethanol, no difference could be observed in the precipitation experiments. Treated human serum as well as treated immune serum produced the same lines as untreated sera against protein A on agar gel diffusion. Neither was any reduction of the ring test titres observed after treatment. The indirect haemagglutination test was still positive, but the titres were reduced to about 1/16 of those of untreated sera. Whether or not iodoacetamide was added to the mercaptoethanol treated sera made no difference to the serological reactions.

The crude cold water extract showed the same serological activities as our protein A preparation (15).

DISCUSSION

Although a single sedimentation boundary was observed during ultracentrifugation, some heterogeneity of our protein A preparation was demonstrated by the increase of D_{app} (16). The Trautman plot which gave a straight line indicates, however, that the heterogeneity is small (17), probably due to minor molecular variations within the preparation. The broad zones of serologically active material found by electrophoresis and the variation in the titres of the eluates may also indicate a certain degree of heterogeneity.

As our protein A preparation exhibits three separate serological activities, the possibility of at least three different types of molecules exists.

The sensitizing ability of protein A is the only activity lost by carboxypeptidase treatment (4). The small part of the preparation digested by this enzyme thus seems to be responsible for the sensitizing ability. Assuming complete digestion, the sensitizing substance constitutes a maximum of 3 per cent of the total weight. The major part of the material should then constitute the precipitinogens which apparently are open polypeptide chains, as indicated by their content of simple amino acids and their relative stability to heat.

The slow sedimentation of protein A in the ultracentrifuge rendered the measurement of the sedimentation coefficient somewhat uncertain. Good agreement was, however, obtained for the diffusion coefficients.

determined from the sedimentation and diffusion studies independently. By the approach to equilibrium run an average molecular weight of 12,200 was found. This value is nearly the same as that found for a similar preparation isolated by Yoshida *et al.* (18).

A small peptide, not attached to the precipitinogens, would influence D_{app} even if it did not leave the meniscus on ultracentrifugation. However, if the peptide were free it most probably would have been separated from the precipitinogens by ion-exchange chromatography and/or electrophoresis. It is, therefore, reasonable to believe that it is attached or adsorbed to the precipitinogens in the purified preparation, though some of the sensitizing antigen apparently is free in the crude extract (6). On column chromatography of a crude extract (6), a fraction showing only the sensitizing ability was obtained. Hydrolysis and paper chromatographic analysis of this fraction showed the amino acids lysine, aspartic acid (trace), serine, glycine, glutamic acid and alanine (weak). Of the amino acids released by carboxypeptidase, valine and leucine thus seem to belong to the precipitinogens, whereas it is uncertain whether or not the aspartic acid and all of the alanine derive from the sensitizing component. Table 2 shows that except for alanine, only one amino acid residue of those derived from the sensitizing principle is released per molecule (M_w 12,000) by carboxypeptidase. If complete digestion with this enzyme were obtained, the sensitizing antigen should be a hexa- or hepta-peptide attached to the precipitinogens, constituting only 5 to 6 per cent of total weight of protein A.

The linkage between precipitating and sensitizing antigens is obscure. Since carboxypeptidase is active, a linkage affected through the C-terminal amino acid of the sensitizing antigen is excluded. The linkage is apparently labile. As with polysaccharide A (15), tanned sheep erythrocytes absorb all sensitizing antigen from a protein A solution where the precipitating ability is unaffected. The adsorption to tanned sheep erythrocytes is probably of electrostatic nature.

The number of free amino groups in each molecule (M_w 12,000) amounts to 16. This is compatible with 15 ϵ -amino groups of lysine and one terminal α -amino group of the chain. Previous experiments (4) showed small amounts of ϵ -DNP-lysine, and the only N-terminal amino acid observed was alanine. An increase in the number of free amino groups corresponding to 15 per molecule was observed after tryptic digestion. This observation agrees completely with the calculated number of lysine residues per molecule and all the ϵ -amino groups of lysine are apparently free. The small amount of ϵ -DNP-lysine observed must be due to failure in the isolation procedure rather than to blocking of the ϵ -amino group by a substitute as previously suggested (4).

Treatment of immune serum with mercaptoethanol at neutral pH results in conversion of the macroglobulins into smaller molecular entities (1) with loss of the antibody activity due to these macroglobulins, while the γG globulins are not affected. No differentiation of the

precipitins against protein A was obtained by mercaptoethanol treatment of sera treated and untreated sera giving the same lines and the same ring test titres. The precipitins thus seem to be of the 7S type. In the indirect hemagglutination test the titres were reduced indicating antibodies against the sensitizing substance of 7S γ globulins as well as of 19S γ globulins.

Our protein A preparation seems to be composed of two closely related molecular entities, i.e. two open polypeptide chains each having a specific precipitating ability and small side-chains with the ability to sensitize tanned sheep erythrocytes. The side chains are probably hexa- or hepta-peptides whereas the precipitating entities each contains about 120 amino acid residues. The main chains as well as the side-chains are digested by trypsin and all the serological activities are destroyed. Carboxypeptidase destroys the sensitizing ability only in the case either that proline is near the C-terminus of the precipitinogens or that attack by this enzyme is sterically hindered. Since some free sensitizing principle has been found in crude extract (6) it is possible that not all precipitinogens contain side-chains. If so this could give rise to the observed heterogeneity.

It is tempting to assume that the serological activities of protein A are due to certain amino acid sequences. Different extraction methods may give preparations with various molecular size exhibiting the same serological activities.

Yoshida *et al.* (18) prepared their antigen by dornase digestion of undigested cell wall and observed one line only on agar diffusion against immune serum. This line corresponded to the line produced by normal human serum. The molecular weight of their preparation agrees however very well with that found in the present study. *Leifkint & Sjöquist* (12) have recently extracted bacteria in the cold and isolated their protein A antigen from the supernatant after acid precipitation. This preparation (fraction A 1) showed the same serological properties as our protein A (10) whereas the sedimentation constant reported ($s_{20}^w = 2.6$) (12) differs greatly from that found in the present experiments as well as from that reported by Yoshida *et al.* (18).

Boiling is a drastic way of extracting antigenic material but compared to the cold water extracted material all the serological activities are retained. As the main interest is to study the primary protein structure it is an advantage to work with the simplest units showing the serological activities. Extraction of protein A by boiling is preferred because this procedure most probably gives simpler units than extraction by cold water.

SUMMARY

The homogeneity, molecular size and shape of protein A have been studied.

No separation of the serological activities was observed on electrophoresis.

Ultracentrifugation experiments showed a small molecular heterogeneity and an average molecular weight of about 12 000 was found.

Carboxypeptidase digestion destroys the sensitizing but not the precipitating activity. Quantitative estimation of the amino acids released by carboxypeptidase and compared to those found in a column fraction having sensitizing but not precipitating ability shows that the sensitizing component constitutes a small part of the preparation (5 to 6 per cent). Most likely it is a hexa- or heptapeptide linked as a side chain to the precipitinogens which are open polypeptide chains of about 120 amino acid residues.

The observed heterogeneity on ultracentrifugation may be due to the absence of the sensitizing factor in some of the precipitating molecules.

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M. XENOPEI

A Bacteriological Study of *M. xenopei* including Case Reports of Danish Patients

By

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Mycobacterium xenopei, a newly described pathogen, has been recognized in specimens from four Danish patients. *Mycobacterium* other than *tuberculosis* are isolated frequently from sputum in Denmark in 1960-61 constituting about 10 per cent of all mycobacteria identified. Only a small number of such bacteria appear to have been pathogenic for the patients concerned. These include *M. avium*, the closely related Bittex group, *M. scrofulaceum* and *M. fortuitum* (6, 7, 10, 9).

First described by Schwabacher in 1959, *M. xenopei* was recognized to be probably pathogenic for man by Marks & Schwabacher in 1963. Furthermore, strains of this species have been identified by Manton in Holland (1963) and Boursvert in France (1963). A few of these strains were isolated from resected pathological lung tissue after having been found in the same patient's sputum. Though the relationship to tuberculosis-like disease is less thoroughly established, many of the isolates have been considered to be of clinical significance. The appearance of the species in urine has also been reported.

The present study includes case reports of four Danish patients and a bacteriological study of the four Danish strains together with eight received from England. The three from Mark's were classified by him as *M. xenopei*. The five from Vassau were part of a number of strains sent to Magnusson and were supplied by the latter for use in the present study because they formed a homogeneous group in his experiments.

A CASE HISTORIES

The following case reports are based on information provided by the hospitals and dispensaries where the patients were diagnosed and have

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been treated. Comparative intradermal tests (Mantoux) were performed in March 1966 on two of the patients (Nos 3 and 4) with sensitizing produced from the following mycobacteria: *M. tuberculosis* (RT 23) *M. avium* (RS 10) *Ralley organism* (RS 23) *M. scrofulaceum* (RS 95) and *M. xenopet* (RS 631). The doses were RT 23 0.02 µg/0.1 ml = 1 TU other preparations 0.1 µg/0.1 ml = 1 TU. The antigens were applied simultaneously on both fore arms, the reactions read after 72 hours and registered as the diameter of induration in millimetres.

Case No. 1. H₂ male born 1903 former porcelain worker. The patient was employed previously at a porcelain factory and has a history of silicosis dating from 1945 when he was hospitalized for the first time. Since 1947 he has been under constant observation and treatment for that disease. Roentgenograms of the lungs showed large infiltrations and cavities. The patient is severely handicapped by his disease and has been in hospital repeatedly. N. BCG vaccinati m Mantoux test 1962: 1 TU human tuberculin 10 mm (72 hours).

Sputum. Numerous examinations by culture since 1945. No isolation of tubercle bacilli but since 1956 *M. xenopet* is isolated repeatedly with numerous innumerable colonies. As regards the bacteriological examinations see below (SSC 86).

Diagnosis. Pneumonia silicosis pulmonis (silicosis).

Treatment. Chemotherapy against the mycobacteria but treatment with penicillin and sulphamides at intervals.

Case No. 2. N male born 1894 married clerical worker formerly blacksmith. Included in routine screening at the tuberculous dispensary up to 1957 with normal findings. Vaccinated with BCG. On the whole well until December 1960 when it was found that he was suffering from a malignant tumour in the rectum. Laparotomy and abdominal hysterectomy performed with uncomplicated course. During hospitalization a cavity in the upper lobe of the right lung corresponding to the space II was discovered. Silicosis pneumoniae thoracis on the right side disappeared completely after treatment. Three gastric lavage specimens gave negative results for tubercle bacilli and three sputum specimens were negative by microscopy. Discharged from hospital in February 1961 and followed up at the tuberculous dispensary. Repeated roentgenological examinations there showed decrease in the infiltration and cysts and bronchiectasis in the right lung. By April 1963 a large cavernous infiltration had developed in the middle area of the left lung. Symptoms during the previous few months were fatigue and increasing dyspnoea and the temperature was partially elevated. The patient was admitted to the Tuberculous Department of Frederiksborg Hospital from April 30 to June 15, 1963. S₁ 20 mm per hour. Haemoglobin percentage 107. Mantoux test 1 TU human tuberculin 8 mm (72 hours). On admission to hospital his general condition was good but he was thin and had functional dyspnoea. After discharge from hospital the patient treatment and follow-up.

Sputum. Microscopy consistently negative. No isolation of tubercle bacilli but since January 1962 *M. xenopet* demonstrated by culture in six sputum specimens with few to innumerable colonies. Last examination in September 1964. As regards the bacteriological examinations see below (SSC 780).

Diagnosis. Pneumonia (atypical mycobacteria). Thoracic malignancy. Neoplasm of rectum.

Treatment. SM 1 AS + INH from May 31 August 8, 1963. Total dosage SM 33 g PAS 1278 g INH 282 g SM + 1 AS + INH from April 29 to September 11, 1964. T₁ 1 g T₂ 1 g T₃ 1 g T₄ 1 g T₅ 1 g T₆ 1 g T₇ 1 g T₈ 1 g T₉ 1 g T₁₀ 1 g T₁₁ 1 g T₁₂ 1 g T₁₃ 1 g T₁₄ 1 g T₁₅ 1 g T₁₆ 1 g T₁₇ 1 g T₁₈ 1 g T₁₉ 1 g T₂₀ 1 g T₂₁ 1 g T₂₂ 1 g T₂₃ 1 g T₂₄ 1 g T₂₅ 1 g T₂₆ 1 g T₂₇ 1 g T₂₈ 1 g T₂₉ 1 g T₃₀ 1 g T₃₁ 1 g T₃₂ 1 g T₃₃ 1 g T₃₄ 1 g T₃₅ 1 g T₃₆ 1 g T₃₇ 1 g T₃₈ 1 g T₃₉ 1 g T₄₀ 1 g T₄₁ 1 g T₄₂ 1 g T₄₃ 1 g T₄₄ 1 g T₄₅ 1 g T₄₆ 1 g T₄₇ 1 g T₄₈ 1 g T₄₉ 1 g T₅₀ 1 g T₅₁ 1 g T₅₂ 1 g T₅₃ 1 g T₅₄ 1 g T₅₅ 1 g T₅₆ 1 g T₅₇ 1 g T₅₈ 1 g T₅₉ 1 g T₆₀ 1 g T₆₁ 1 g T₆₂ 1 g T₆₃ 1 g T₆₄ 1 g T₆₅ 1 g T₆₆ 1 g T₆₇ 1 g T₆₈ 1 g T₆₉ 1 g T₇₀ 1 g T₇₁ 1 g T₇₂ 1 g T₇₃ 1 g T₇₄ 1 g T₇₅ 1 g T₇₆ 1 g T₇₇ 1 g T₇₈ 1 g T₇₉ 1 g T₈₀ 1 g T₈₁ 1 g T₈₂ 1 g T₈₃ 1 g T₈₄ 1 g T₈₅ 1 g T₈₆ 1 g T₈₇ 1 g T₈₈ 1 g T₈₉ 1 g T₉₀ 1 g T₉₁ 1 g T₉₂ 1 g T₉₃ 1 g T₉₄ 1 g T₉₅ 1 g T₉₆ 1 g T₉₇ 1 g T₉₈ 1 g T₉₉ 1 g T₁₀₀ 1 g T₁₀₁ 1 g T₁₀₂ 1 g T₁₀₃ 1 g T₁₀₄ 1 g T₁₀₅ 1 g T₁₀₆ 1 g T₁₀₇ 1 g T₁₀₈ 1 g T₁₀₉ 1 g T₁₁₀ 1 g T₁₁₁ 1 g T₁₁₂ 1 g T₁₁₃ 1 g T₁₁₄ 1 g T₁₁₅ 1 g T₁₁₆ 1 g T₁₁₇ 1 g T₁₁₈ 1 g T₁₁₉ 1 g T₁₂₀ 1 g T₁₂₁ 1 g T₁₂₂ 1 g T₁₂₃ 1 g 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was variable but, on the whole poor. The patient did not wish to be admitted to hospital and could not tolerate chemotherapy. He died in his home in March 1965.

Case No 3 M male born 1919 married caretaker. Previously healthy. Never employed in any occupation likely to cause chronic lung processes. Vaccinated with BCG in 1947. In 1952 took part in routine screening at the tuberculosis dispensary. Two months before admission to hospital increasing fatigue, poor appetite and loss of weight, pain in the right side of the chest, increasing cough and expectoration, temperature about 38°. Referred to the tuberculosis dispensary because of these symptoms. Roentgenogram showed right sided dense infiltration in the space I III. Admitted to the Tuberculosis Department of Bispebjerg Hospital in October 1963. On admission the temperature was slightly elevated during the first few days but then became normal. Discharged in May 1964 subsequently out patient treatment. Roentgenogram and tomogram showed a large infiltration through the upper part of the right lung from front to back. No definite cavities. Remainder of the right lung and left lung normal. S.H. 56 mm per hour. Haemoglobin percentage 14.5. Blood pressure 150/90. Urine normal. Mantoux test 1 TL human tuberculin III mm (72 hours).

Sputum. Microscopy revealed acid fast rods resembling tubercle bacilli. Culture during the first six weeks gave growth four times of the same kind of mycobacteria (*M. xenopet*) with moderate few numerous and few colonies respectively. Subsequently culture was consistently negative. Tubercle bacilli never recovered. As regards the bacteriological examination see below (Nst 993).

Diagnosis. Infiltr. pulm. dxt. Cystis pulm. dxt.

Treatment. S.H. from November 1 1963 to May 1 1964. Total dosage 81 g.

PAS from November 1 1963. Still being treated 1968.

NH from November 1 1963. Still being treated 1968.

The lung processes decreased quite quickly during treatment and became less dense but there were still infiltrations in the upper part of the right apex when examined in September 1965. The patient was allowed to work full time from August 1964 but reports for examination every month while under out patient treatment.

Comparative intradermal tests performed in March 1966 gave the following results: 1 TL human tuberculin RT 23 2 mm, 5 TL asian tuberculin RT 10 no reaction, 5 TL Battey sensitin RS 23 no reaction, 5 TL *M. scrofulaceum* sensitin RS 9a no reaction, 5 TL *M. xenopet* sensitin RS 631 20 mm.

Case No 4 L male born 1900 married foundry worker (ceased employment 1951). Since 1949 roentgenological processes in lungs (? silicosis). Extensive calcification in hilus. Sputum examination 1963 no tubercle bacilli by culture. Admitted to County and Municipal Hospital, Nærbjerg Mors, in May 1963 on account of increasing tenderness and swelling of the right scrotum. A round smooth 1.5 cm tumour the size of a goose egg was found corresponding to the right testis. S.H. 40 mm per hour. Haemoglobin percentage 84. Blood pressure 140/75. Microscopy of urine normal.

Diagnosis. Epididymitis.

Treatment. Hemicastration dxt.

The testis was macroscopically normal and the tunica vaginalis enlarged. There were small foci containing greenish pus in the epididymis. Histological examination revealed epididymitis chr subacute m gr. Slight suspicion of tuberculosis. Perorchitis chr m gr. No chemotherapy. Post-operative course uncomplicated. Two out patient follow up examinations a few months later showed normal recovery. No fistula formation or orchitis. Bacteriological examination of pus from the epididymis gave growth of *M. xenopet* with numerous colonies. For further details see below (Nst 731).

Comparative intradermal tests performed in March 1966 gave the following results: 1 TL human tuberculin RT 23 no reaction, 5 TL asian tuberculin RT 10 no reaction, 5 TL Battey sensitin RS 23 no reaction, 5 TL *M. scrofulaceum* sensitin 3 mm, 5 TL *M. xenopet* sensitin RS 631 12 mm.

A summary of the case histories is given in Table 1.

TABLE I
 M xenoid Summary of Case Histories

Case	Sex	Age (years)	Disposition	Localization of disease	Staining material	Pathogenic	Treatment	Response to treatment	Outcome of disease
1	M	52	Silicosis	Lungs	Sputum	Ductal	None		Severely complicated
2	M	62	Malignant tumor of the rectum	Lungs	Sputum	Yes	Chemotherapy	Transient	Decreased
3	M	44	None	Lungs	Sputum	Yes	Chemotherapy	Yes	Recovered
4	M	63	None	Thymus	Yes	Yes	Surgery	Yes	Recovered

* Age at time of first isolation of M xenoid

■ BACTERIOLOGICAL STUDIES

Table 2 gives a survey of the strains studied and their origin.

Isolation of bacteria. All specimens from the Danish patients were inoculated primarily on Lowenstein-Jensen medium after treatment with 4 per cent sodium hydroxide. Growth appeared on these primary cultures for the first time after five to six weeks incubation (37°). Most of the English strains were isolated from specimens treated with sodium hydroxide (Marks 1966; Nassau 1966).

TABLE 2
M. xenopetis: Origin of Cultures

Origin	Original designation	Source	SSC No.
Denmark	Case 1	Sputum	786
	Case 2	Sputum	780
	Case 3	Sputum	989
	Case 4	Urus (epithelium)	791
England	Nassau TB 24	Sputum	991
	Nassau TB 38	Urine	982
	Nassau TB 40	Sputum	983
	Nassau TB 65	Gastric lavage	984
	Nassau TB 82	Urine	985
England	Marks 9889	Sputum	990
	Marks 13806	Sputum	997
	Marks 31422	Sputum	988

* SSC = Statens SerumInstitut Collection.

Standard cultures. All the strains were grown in Dubos fluid medium containing Tween 80 and adjusted in the Klett-Summerson photometer to 55-60 corresponding to about 1 mg. semi-dried bacilli per ml. The number of bacterial units in these suspensions was determined to be about 10 per ml. by colony counts on Lowenstein-Jensen medium. All the experiments described subsequently were performed with the standard culture or subcultures from it.

Colony morphology and pigmentation. Well isolated colonies of different strains on Lowenstein-Jensen medium resembled each other closely. After about three weeks growth at 37° the colonies were dog-eared, dome-shaped without any marginal zone, smooth and with yellow to pale pink pigmentation. However, SSC 989 showed more berry-shaped colonies with slightly rough surface. Only one kind of colony was found on both medium and on Dubos oleic acid albumin agar without pyruvate.

The effect of light on the pigmentation was studied both as suggested by Runge (1955) and by exposure to daylight (from first growth) at room temperature. All the cultures were eight weeks old.

There was no change in pigmentation after short or long exposure to light. The colonies on Lowenstein-Jensen medium were butyrous and moist and emulsified easily into a finely dispersed suspension. Stained bacteria were not decolorized by alcohol and acid and were long and slender.

TABLE 3
M. xenopi Drug Resistance

(Figures indicate highest concentration in γ ml of drug permitting quantitatively similar growth as in control tube. Medium: Lowenstein-Jensen)

SSC No	SM	PAS	Thiosemi- carbazone (TM)	INH	Vio- mycin	Cyelo- serine	Pyrazin- amide	Ethion- amide (1314)
786	2	16	≥ 25.6	0.04	c	c	c	c
780	c	0.4	≥ 25.6	0.04	c	c	1000	25
939	c	0.4	≥ 25.6	0.04	c	c	1000	c
791	2	0.4	≥ 25.6	0.04	c	c	1000	c
931	2	0.4	≥ 25.6	0.04	c	16	c	c
932	c	c	≥ 25.6	0.04	c	c	c	c
983	2	16	≥ 25.6	0.32	c	16	c	c
934	c	0.4	≥ 25.6	0.08	c	c	100	c
985	c	0.4	≥ 25.6	0.04	c	c	200	c
936	c	c	≥ 25.6	c	c	c	c	c
987	2	0.4	≥ 25.6	0.04	c	c	100	25
988	c	0.4	≥ 25.6	0.04	c	c	1000	c
Control strain (No. 5)	1	0.1	0.1	0.02	10	8	10	8-16

c = growth as with control strain (*M. tuberculosis*)

— n l examined

Resistance determinations were made on Lowenstein-Jensen medium to which the different drugs were incorporated before coagulation. The tests on Lowenstein-Jensen medium were performed with standard culture diluted 100 times, each tube inoculated with 0.1 ml. The tubes were placed horizontally until the next day when they were sealed with paraffin and incubated in vertical position at 37°. The results were read and recorded after three weeks. A highly sensitive culture of *M. tuberculosis* (Strain No. 5) was used as the control strain. The figures in Table 3 show the highest concentration of the various drugs at which the bacteria were able to grow to the same extent as on the control tubes, i.e. with 100 or more colonies per tube. Almost all the strains of *M. xenopi* were sensitive to streptomycin, viomycin, cycloserine and ethionamide (1314). The degree of resistance to PAS and INH varied slightly but the values were never high. There were large variations from strain to strain with pyrazinamide. Repeated experiments with the same strains indicate that these differences were due to technical difficulties encountered when working with that drug. In these cases the

highest value is given in Table 3. Only to thiosemicarbazone (TSC) were all the strains strongly resistant. Furfuryl 2 hydrazine (not shown in the table) had no influence on the growth as tested at 10, 20 and 40 $\mu\text{g/ml}$ (Bönteke 1958). 100 units of penicillin per ml in Dubos oleic-acid albumin agar caused strong inhibition of all strains, although with four of the strains a small percentage of the bacterial units were able to grow.

The effect of temperature was determined by examining tubes of Löwenstein-Jensen medium inoculated with 0.1 ml of dilutions of the standard culture incubated at 22°, 37°, 40°, 43° and 45°. Reading and recording were made after 1, 2, 3, 4 and 8 weeks. The results in Table 4 refer to the first time (in weeks) at which well isolated colonies were visible. No growth after eight weeks is recorded as 0. All the strains grew at 45° but not at 22°, even after an observation period of eight weeks. At temperatures of from 37° to 45° there was no systematic difference in the time at which growth was first observed. Results not given in the table show that this also applied both as regards the maximum number and the size of the colonies.

TABLE 4

M. xenopi. Effect of Temperature on Growth on Löwenstein-Jensen Medium

SSC No	First visible growth (weeks)*				
	22°	37°	40°	43°	45°
786	0	2	2		2
780	0	2	2		2
939	0	2	2	2	2
791	0	2	2	2	2
991	0	3	3	3	3
992	0	3	3	3	3
983	0	4	4	4	4
994	0	3	3	3	3
995	0	3	3	2	2
996	0	4	4	4	4
997	0	3	3	3	3
999	0	3	3	3	3

0 = no growth after eight weeks

* See footnote p. 591

= not examined

BIOCHEMICAL TESTS

Methods

Amilase test (Bönteke 1960). About 100 mg of culture was scraped from Löwenstein-Jensen slants and transferred into a previously weighed tube. The bacterial mass was comminuted with a glass rod and sterile saline added gradually. The bacteria were then washed twice with 10 ml of sterile saline and emulsified in phosphate buffer (pH 7.2) to a concentration corresponding to 10 mg per ml (five tenths ml of each). Ten amilase units (0.001 U/ml) was placed in a centrifuge tube and 0.5 ml of the bacterial suspension added simultaneously. One drop of culture was inoculated on a blood agar plate as control for contamination. The

centrifuge tubes were placed in the incubator at 37° C for about 19 hours. The test was then performed by adding manganese sulphate then 1 ml of pyridine as described by H. A. A. After filtering for 15 minutes the tubes were centrifuged at 3000 r.p.m. for 15 minutes and the colour of the supernatant measured in the Coleman Junior spectrophotometer at a wave length of 535 mμ. The direct concentration of ammonia was determined by reference to a standard ammonia curve. The degrees of reaction (y per ml) were recorded as follows: 0 < 2 -; > 2 +; 5 + + > 5 to 10 + + + > 10. The amides are numbered as suggested by H. A. A. as follows: 1 Acetamide 2 Benzamide 3 Carbamide 4 Nicotinamide 5 Nicotinamide 6 Pyrazinamide 7 Naloxamide 8 Allantoin 9 Succinamide 10 Malonamide.

Phenolphthalein sulphate test (Wayne modification 1962): 10 ml of Dubos fluid medium with Tween 80 in a Coleman spectrophotometer cuvette was inoculated with 0.1 ml of a tenfold dilution of standard culture and incubated at 37°. Suspension of the bacteria was facilitated by using a 15 mm diameter plastic sealed magnetic bar in the cuvette. The growth was measured at intervals of a few days until an optical density of 0.100 at 540 mμ was obtained. A sterile solution of potassium phenolphthalein disulphate was then added to a final concentration of 0.001 M. Incubation was repeated at 37°. Samples of 0.5 ml were taken after 3 days and after 1, 2, 3 and 4 weeks and equal parts of a 1 M sodium carbonate solution added. The hydrolysis of the phenolphthalein was evaluated by means of the resulting pink colour and recorded after 30 minutes in five grades from (-) to + + + +.

Semiquantitative catalase test (Wayne 1962): Laewenstein-Jensen medium was coagulated in test tubes placed in upright position. The surface was inoculated with 0.1 ml of a tenfold dilution of standard culture. An aluminium cap was placed loosely on the tube which was then incubated at 37° for three weeks. 1 ml of a mixture of equal parts of 10 per cent Tween 80 and 30 per cent hydrogen peroxide was added. When foaming reached its maximum the height of the foam column was measured in millimetres and recorded as follows: + for a few bubbles to 5 mm + + > 5 to 10 + + + > 10 to 15 + + + + > 15 to 20 + + + + + > 20.

Nicotinic acid test: Since large fully grown colonies are the most suitable for this test four to five week old cultures on Laewenstein-Jensen medium were used. In order to promote growth the tubes had been closed for two to three weeks with loosely fitting aluminium caps. About 3 ml of sterile 0.9 per cent sodium chloride solution was added and the tube placed in boiling water for one hour. After cooling 1 ml sample was taken and the Lehnke test performed by adding 1 ml of 1 per cent aqueous potassium cyanide solution and immediately afterwards 1 ml of 5 per cent chloramine solution. If the mixture was opaque it was centrifuged before the reaction was read. After five minutes the yellow colour was measured in the Coleman spectrophotometer at 460 mμ. The amount of nicotinic acid was determined by reference to a standard nicotinic acid curve.

Tween 80 degradation test (Wayne modification 1962): One loopful of a three to four week old culture was taken from Laewenstein-Jensen medium and emulsified in 4 ml of 1.5 M phosphate buffer pH 7.0 with 0.5 per cent Tween 80. Neutral red was added as indicator. The bacterial suspension was incubated at 37°. Degradation by Tween causes the fluid to become red as acid is liberated. Observations for change of colour was performed after four hours. If there was no reaction at that time the observation was repeated once a day for seven days.

Nitrate reduction test (Wayne modification 1962): The bacteria were grown in a Coleman spectrophotometer cuvette (as described) for the phenolphthalein sulphate test. In 10 ml of Dubos fluid medium with Tween 80 containing 0.01 M sodium nitrate. When the required optical density was reached an aliquot of 0.1 ml was taken and added to 8.5 ml of a 0.006 per cent solution of 2,6-diaminonaphthalene. 0.1 N hydrochloric acid 1.5 ml of 0.02 per cent 1-(1-naphthyl) ethylene diamine dihydrochloride solution was then added and the optical density measured after five minutes at 540 mμ. The concentration of nitrate in the sample was determined by reference to a standard nitrite curve.

Results

Details of the experimental results are shown in Table 5. It is of special interest that the reactions of amides 5 (nicotinamide) and 6

TABLE 5
Various Biochemical Tests

Strain	3°	Amlase reaction	Others	Phenolphthalein sulphatase Reaction	Day	Catalase	Niacin γ/ml	Tween degradation Reaction	Day	Nitrate reduction γ/ml
80	+	+	0	++	3	+	0.9	0	7	20
780	+	++	0	+++	3	+	0	0	7	10
189	+	+	0	+++	3	++	1.1	0	7	10
791	+	+	0	+++	3	++	1.8	0	7	10
921	+	+	0	+++	3	+	0.1	0	7	0
982	+	+	0	+++	3	+	0.7	0	7	0
983	+	+++	0	+++	3	+	1.6	0	7	0
984	+	+++	0	+++	3	+	0.7	0	7	0
995	+	+++	0	+++	3	+	0.7	0	7	0
996	+	++	0	++	3	+	1.5	0	7	0
997	+	++	0	+++	3	+	1.4	0	7	0
998	+	++	0	+++	3	+	2.2	0	7	0

* 3° = Nicotinamide † 6° = Pyrazinamide

(pyrazinamide) were positive that the sulphatase reaction occurred rapidly and was generally strongly positive and that the catalase reaction was only weak.

D. PATHOGENICITY AND VIRULENCE

Methods

Experimental animals. *Hens* (weight 2100-2400 g) and *rabbits* (weight 1900-2100 g) infection dose 5 mg intravenously—in a filter in with one of the strains (SSC 71) 1 and 10-2 mg intravenously—observation time 3 months. The hens showed a reaction to avian tuberculin before infection. *Culmex pigs* (weight 275-375 g) infection doses 1 mg intravenously, 1 mg intraperitoneally, 0.1 mg intracutaneously in two places—observation time 2 months. *Mice* (weight 18-20 g) Albin strain (C) black mice (C₅₇) and red mice (Clethrionomys glareolus Schreb.) infection dose 10 mg intraperitoneally—observation time 2 months.

Recording. Macroscopic changes in the visceral organs of all animals and in rabbits any processes occurring in the joints and tendon sheaths of the legs were recorded as 0, +, ++, +++. The local reactions in guinea pigs after intracutaneous infection were measured and evaluated after 1, 2, 3, 4 and 8 weeks. Smears were made from liver, spleen and lungs of all the mice and some of the other animals and stained by the Ziehl-Neelsen method with 3-4 per cent aqueous picric acid as contrast stain. The numbers of bacteria were recorded as + 1-50, ++ 51-300, +++ 2-70 per individual, 4 immersion sight field, ∞ innumerable. Observation time four minutes. In the mouse experiments the number of bacteria in the smears are expressed in terms of the following index—

- 0 — no bacteria
- 1 — 1-50 bacteria
- 2 — 51-300 bacteria
- 3 — 2 per sight field to ∞ bacteria

The figures in Table 11 show the distribution of the twelve strains of *M. xenopoli* according to the average index size. The average index is worked out per organ per mouse and shown separately for the three mouse breeds.

Culture was performed on tissue from all the animals except the mice. Before inoculation the material was comminuted in mortar and treated with 4 per cent NaOH. Inoculation was made on 5-ur tubes of Löwenstein-Jensen medium which were incubated at 37°. The results of culture were registered as follows according to the number of colonies per tube: + 1-10, ++ 11-49, +++ 50-99, ∞ \geq 100.

Results

Hens. The strains were inoculated into 16 hens. It will be seen from Table 6 that 12 of the animals died in the course of the observation period: the first on the 30th and the last on the 75th day. Eight animals had macroscopically visible lesions in spleen (mainly enlargement), seven in liver and one in lungs. Culture revealed large numbers of bacteria in one or more organs in all the animals (except one) regardless of time of death and extent of macroscopically visible lesions. However, microscopic examination of smears showed relatively small numbers of bacteria.

Titration of the virulence with one of the strains (SSC 71) is shown in Table 7. The survival times were dependent on dosage. With 10 mg all three hens survived the whole observation period. With 5 and 1 mg the spleen was enlarged in all animals except one and innumerable

TABLE 6
Mycoplasma parvum Determination - Hens (5 mg 1's)

No.	Day of death or killed (3 mths)	Spleen		Liver		Kidney		Lung	
		Culture	Microscopy Acid fast bacilli	Culture	Microscopy Acid fast bacilli	Culture	No of colonies	Culture	Microscopy Acid fast bacilli
786	30	∞		∞		+++	+++	+++	
787	42	∞		∞			∞		
788	51	∞		∞		+	∞		0
789	75	∞	+	∞	∞		∞		
790	37	∞		∞			cont		
791	k	++	0	∞+	0	0	∞	0	0
792	k	++	0	∞+	++	0	∞	0	0
793	k	0	+	∞	+	0	0	0	0
794	k	∞	+	∞	+	+	+	+	0
795	75	∞	+	∞	0	∞	∞	+	+
796	k	+	0	∞	0	++	+	0	0
797	56	∞	0	cont	0	++	++	0	0
798	78	∞	0	∞	0	++	cont	0	0
799	56	∞	+	∞	0	++	∞	0	0
800	46	∞	∞	∞	+	++	+	0	0

— not examined

cont — contaminated

colonies were recovered from one or more organs in all cases. With 10⁻⁶ mg there were no microscopically visible processes and only one of the hens had large numbers of bacteria in the organs.

Rabbits. The strains were examined in 17 rabbits. It will be seen from Table 8 that all the rabbits except two survived the observation period. The rabbit which survived only 23 days presumably died of

TABLE 7
M. xenopel Titration of Virulence in Hens with SSC 791

Dose i.v.	Day of death or killed (3 mths)	Spleen	No. of colonies i.v. culture Liver	Lung
5 mg	22	∞	∞	∞
	23	∞	∞	cont
	24	∞	∞	∞
	33	∞	∞	∞
	k	∞	+	+
1 mg	41	∞	∞	∞
	54	∞	∞	∞
	40	∞	∞	∞
	27	∞	∞	+++
	47	∞	+++	∞
10 ⁻⁶ mg	k	0	0	0
	k	0	0	0
	k	∞	∞	+

The table includes the results of four separate experiments.
cont = contaminated

TABLE 8
M. xenopel Virulence Determination Rabbits (5 mg i.v.)

SSC No	Day of death or killed (3 mths)	Spleen	No. of colonies i.v. culture Liver	Kidney	Lung	Joint
786	66	+	++		+	0
780	k	0	∞		0	0
989	k	+	+	0	+	0
791	23	+++	++		+++	0
	k	+	++	+	++	0
991	k	+	+	0	+	0
982	k	0	+	0	+	+
983	0	+	++	+	+	0
984	0	0	+	+	+	0
985	k	0	+	0	+	0
986	k	+	++	0	+	0
987	k	+	+	0	0	+
988	k	+	+	0	+	0

0 = not examined

intercurrent disease, since six other rabbits infected with the same strain all survived the infection (Tables 8 and 9). There were only few macroscopically visible lesions in the organs and the joints and tendon sheaths were not affected. Culture gave growth of far less colonies than in the above mentioned experiment on hens. From only two animals could a few colonies be isolated from joints.

Titration of virulence for rabbits was also undertaken with strain SSC 791. The results of culture (Table 9) show that the number of bacteria recovered from the organs was far less than in the hen experiment.

TABLE 9
M. xenopei Titration of Virulence in Rabbits with SSC 791

Dose 1x	Day of death or killed (3 mths)	Spleen	No. of colonies by culture Liver	Lung	Joint
5 mg	k	0	++	0	+
	k	0	+	0	0
	k	0	+	+	+
	k	+	+++	+	0
	b	0	++	+	0
1 mg	k	0	+	0	0
	k	0	+	0	0
	k	0	+	0	+
	k	0	+	0	0
	b	0	+	0	+
10 ⁻⁶ mg	k	0	0	0	0
	k	0	0	0	0
	k	0	+	0	0

The table includes the results of four separate experiments

Guinea pigs The strains were injected intraperitoneally into 20 animals. The macroscopically visible lesions were limited to the site of injection in the form of necrotic nodules in the omentum in about 50 per cent of the animals. Small numbers of bacteria could be recovered from the omentum as well as from one or more organs.

A similar number of animals were injected intravenously. All survived the observation period and only few bacteria could be recovered from one or more organs.

Intracutaneous injection of the bacteria (Table 10) produced erythematous nodules of varying size with maximum ulceration after about four weeks. This could still be seen after eight weeks with five of the strains though generally by then the size of the lesions had decreased considerably and one had healed completely.

Mice The examinations with the three breeds of mice showed that only a small number of animals died after the intraperitoneal injection of 10 mg. The deaths occurred particularly among the house and red

mice, but the findings were not reproducible in repeated experiments with the individual bacterial strains. Autopsy after two months showed practically no macroscopically visible lesions in the organs.

It will be seen from the distributions given in Table 11 that the three breeds of mouse reacted differently to infection with *M. xenopri*. The largest number of bacteria were found in the house mice and the smallest number in the red mice. This applies to all three organs examined. Furthermore, the index for the individual breeds indicates that the number of bacteria was greatest in the spleen and lowest in the lungs. This was confirmed by comparing the results in the individual animals.

TABLE 10

M. xenopri. Size of Local Reactions (mm) after Intracutaneous Injection of 0.1 mg into Guinea Pigs

SSC No	weeks				
	1	2	3	4	8
786	5	14	—	12*	12*
780	5	5	8	8*	3
959	8	11*	15*	16*	9*
791	5	7	10*	11*	6
991	5	6	7	7*	5
982	3	6	8	7	7
983	8	9	7	8*	6*
994	6	7	8*	6*	4*
995	6	7	12*	8*	4*
986	7	7	3	8*	2
997	7	15	16	12*	8
998	8	8	11*	8*	2

* = ulceration

— = not examined

TABLE 11

M. xenopri Mice Experiments
Distribution of 12 Strains According to Average Bacterial Index Calculated per Mouse per Organ (See Text)

Average Index	House mice			White mice			Red mice		
	Spleen	Liver	Lung	Spleen	Liver	Lung	Spleen	Liver	Lung
0	0	0	2	0	2	4	1	4	7
0.1 - 1.4	5	7	10	9	9	8	10	8	5
1.5 - 2.4	4	3	0	3	1	0	1	0	0
2.5 - 3.0	3	2	0	0	0	0	0	0	0

Only the mice which survived the two months observation period are included in the evaluation

Index 0 = no bacteria
1 = 1-50 bacteria
2 = 51-300 bacteria
3 = 2 per sight field > 300 bacteria

F HISTOPATHOLOGY

In connection with some of the autopsies, pieces of tissue were removed from liver, spleen, lung, and kidney, and placed in 10 per cent neutral formalin solution for histopathological examination. Hematoxylin-eosin and acid fast stained sections were examined.

TABLE 12
M. xenopi Histopathological Findings

SSC No.	Day of death or killed (3 mths)	Spleen	Liver	Kidney	Lung	Acid fast bacteria
<i>Hens</i>						
791	35	++++	++++	++	++	Innumerable
982	h	+	++	0	+	Rare
983	h	++	++++	0	++	Innumerable
	k	0	0	0	0	None
984	64	++++	++++	++	+	Innumerable
995	75	++++	++++	0	+	Innumerable
986	h	++++	+++	0	++	Innumerable
	55	+++	++++	+	+	Innumerable
987	38	+++	+++	0	0	Innumerable
	56	++++	++++	+	+	Innumerable
998	46	++++	++++	0	+	Innumerable
<i>Rabbits</i>						
791	h	0	++	0	0	None
991	h	0	0	0	0	None
<i>Guinea Pigs</i>						
791	h	0	+	0	0	None
991	h	+	+	0	0	None
	k	0	+	0	0	None

++++ = Innumerable granulomata with confluence

+++ = Many discrete granulomata

++ = Scattered discrete granulomata

~ = Rare typical granulomata

0 = No recognizable granulomata

— = No tissue

The essential histopathological lesion in the hen was a small non caseating granuloma composed of irregular large epithelioid cells with pale cytoplasm surrounded by a thin rim of lymphocytes and reticulum cells. In heavier infections the granulomata were confluent but retained at least a degree of morphological integrity. No necrosis of any type was observed in the granulomatous lesions though surrounding parenchymal tissues, particularly the liver, might show extensive coagulative necrosis when innumerable granulomata were present. In those parenchymal organs sampled (liver, spleen, lung and kidney) no specific patterns of distribution of the mycobacterial lesions were observed.

Acid fast bacteria were present in the granulomata in almost all

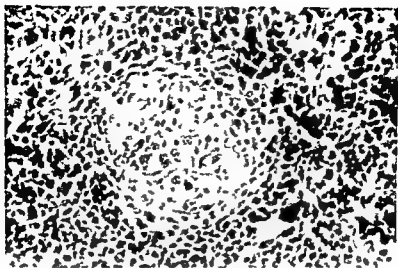


Fig. 1

M. xenopet (SSC 996) (granulomatous lesions in spleen of hen killed after three months (haematoxylin-eosin stain; magnification 420 times)

instances in large numbers and were not seen in other areas. The organisms were long, usually beaded and strongly acid fast. The small but typical granulomatous lesions seen in the liver of rabbits and guinea pigs contained no organisms and are assumed to represent abortive or healing granulomata induced by mycobacterial lipoprotein.

The results of the histopathological examinations are shown in Table 12 and Fig. 1.

DISCUSSION

In the present work the four Danish strains form a homogeneous group indistinguishable from the English strains, three of which have been classified by *Varlt* as *M. xenopet*.

The bacteriological findings generally confirm what has been reported from other sources (15, 16, 1, 13). The bacteria grow slowly with dysgonic colonies of pale to moderate orange colour. There is no growth at 22° but good growth at 45° and the bacteria are sensitive or relatively sensitive to a number of primary and secondary drugs.

Other results of particular interest are the positive reactions to amides 5 (nicotinamide) and II (pyrazinamide) i.e. a pattern said to be characteristic for *M. avium* and strains resembling *M. avium* (*B. nicke* 1960). Furthermore there is moderate to strong positive sulphatase reaction on the third day—a result which has hitherto been recorded only with rapidly growing strains (*M. fortuitum*) (12, 21, 20). The animal experiments reveal a characteristic difference in the ability of *M. xenopet* to multiply in hens and rabbits, both as regards survival time

and recovery of bacteria from the organs. The pathogenicity of the strains for hens has been emphasized recently by *Boister* (1963).

Comparison with *M. avium* is of importance because of a number of mutual characteristics which might give rise to incorrect diagnosis: colony morphology, pigmentation, rate of growth, good growth at 43° weak catalase reaction, and amide pattern. However *M. xenopet* is distinguishable from *M. avium* in a number of ways. Lack of growth at 22° (with *M. avium* almost all strains grow in the course of eight weeks), good growth of all strains at 45° (with *M. avium* about 70 per cent of the strains give growth and of these only about one-fifth give growth of the same number of colonies as at 37°), the rapidly occurring sulphhydrylase reaction and the relative sensitivity to a number of drugs.

As regards the pathogenicity of the strains and their virulence for experimental animals it is the experiments with hens which are of particular interest. Comparison with a number of strains of *M. avium* isolated from pigs shows that *M. xenopet* is somewhat less virulent for hens than *M. avium* even though a dose of 1.5 mg. injected intravenously caused multiplication of the bacteria in the organs and death of many of the animals. The weaker virulence is manifested by the lower lethality, the longer survival time and the finding by microscopy of relatively few bacteria in the organs.

The histopathological findings resemble those observed with *M. avium*. The distribution of lesions is similar to that found with other mycobacteria of low virulence for hens, e.g. strains of the Bitter group.

The difference in the virulence of *M. xenopet* and *M. avium* is evident when tested in rabbits and guinea pigs infected intravenously and in mice infected intraperitoneally. After infection with *M. xenopet* almost all the rabbits survived and it is of special interest that none of them showed signs of chronic processes in particular in joints and tendon sheaths. Such processes are characteristic after infection with *M. avium* and strains resembling *M. avium* (4, 11, 10, 5, 8, 24). The guinea pigs also survived the infection and almost all the animals both rabbits and guinea pigs had only few bacteria in their organs. In contrast in infections with *M. avium* almost all the rabbits die and large numbers of bacteria can be recovered from the organs. About 70 per cent of the guinea pigs die spontaneously with large numbers of bacteria in one or more organs. This applies furthermore to most of the surviving animals. In the mouse experiments also *M. xenopet* is less virulent than *M. avium* of persisting or multiplying in the organs. This is seen with all three mouse breeds and can be demonstrated in spleen, liver and lung.

As regards the pathogenicity of *M. xenopet* for man, patient No. 4 in this study is of particular interest since this is the first case in which it has been demonstrated that *M. xenopet* can provoke extrapulmonary disease. Other reports where information is available concerning the patients mention only pulmonary processes (1, 13, 16). The present

material is too small and non homogeneous to permit the drawing of prognostic conclusions. However the sensitivity of the strains to a number of primary and secondary drugs provides other possibilities of treatment than in infections with *M. avium* and other mycobacteria e.g. strains belonging to Runyon's Group III. Patient No. 3 was apparently cured by means of chemotherapy. In Case No. 2 there may also have been a considerable benefit from treatment which, however, could not be effectuated in full because of hypersensitivity and other circumstances. Surgical removal of the local process without chemotherapy was sufficiently effective treatment in the patient with epididymitis (Case No. 4).

The results of comparative intradermal testing indicate that the sensitin produced from *M. xenopet* can be of assistance in determining whether or not a strain is pathogenic for the patient from whom it was isolated.

With a view to ascertaining the frequency of *M. xenopet* review has been made of a material of mycobacteria other than tubercle bacilli isolated from about 750 Danish patients including all strains which grew at 43° in subculture. It was found that only the strains isolated from the four patients in this study showed the characteristic mosaic of findings. It can be concluded therefore that *M. xenopet* occurs only infrequently in Denmark.

SUMMARY AND CONCLUSION

A description is given of the course of disease in the four Danish patients from whom *M. xenopet* was isolated. In three of these the strain was definitely pathogenic: two provoked lung processes and the third abscesses in the epididymis.

The bacteriological examinations included the four Danish and eight English strains. The study shows that the strains formed a homogeneous group with a characteristic mosaic of properties not observed with previously described species of mycobacteria. On account of a number of similarities with *M. avium* a comparison with that species is included in the discussion.

M. xenopet occurs infrequently in Denmark. It has been demonstrated only in the four patients in this study out of about 750 from whom mycobacteria other than tubercle bacilli have been isolated.

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Subsequent experiments with some of the strains showed that growth at 33-45° occurred one to two weeks earlier than growth at 37-40°.

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CHANGES IN CIRCULATING LYMPHOCYTE POPULATIONS IN PERTUSSIS VACCINATED GUinea PIGS

A Quantitative Study of Blood and Thoracic Duct Lymphocytes in
Normal, Sham Operated and Thymectomized Animals

By

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The importance of an intact thymus for normal morphological and functional development and regeneration of the lymphoid system in rodents is well documented (cf. see *Frnstrom* 1965). The role of the thymus if any during an immune response is not however known. *Eichtelius et al.* (1957, 1958) reported increased cell formation in the rat thymus after treatment with pertussis vaccine thus indicating a contribution of the thymus to the associated lymphocytosis in the blood. *Voller* (1963) and *Metcalf* (1964) nevertheless denied the existence of mitotic stimulation in the thymus during an immune response.

In previous papers we presented evidence of an emigration of small lymphocytes from the thymus. Thus a greater number of small lymphocytes per mm³ of blood was found in the thymic veins than in the afferent arterial blood (*Frnstrom & Larsson* 1966a). An increase in this venous output of small lymphocytes from the thymus could be demonstrated after administration of thyroid hormone (*Frnstrom & Larsson* 1966). The object of the present investigation was to study the cellular emigration from the thymus during an immune response and to compare the response of different categories of blood lymphocytes to antigenic stimulation in sham operated and thymectomized animals. In view of the aforementioned results of *Eichtelius et al.* indicating a possible antigen stimulated emigration of cells from the thymus we used pertussis vaccination as the experimental model.

MATERIAL AND METHODS

Totally 211 guinea pigs with an initial weight of 237 ± 29 g (mean \pm S.E.) were used. The animals were fed on collated turnips carrots and vitamin pellets. They were divided into three main groups i.e. normal rats, sham operated and thymectomized guinea pigs (70, 71 and 70 animals respectively). Operation was performed under ether anaesthesia (subcutaneous infiltration with 0.5 per cent Aveloaine® Aet. using the technique of *Cyfluent* 1953). The animals were in

jected subcutaneously in the back with 0.5 ml of pertussis vaccine (commercial preparation from the State Bacteriological Laboratory, Stockholm Sweden) containing 20×10^6 killed bacteria per ml. In the operated animals infection was performed immediately after sham operation or thymectomy.

In each group 10 guinea pigs were examined 1, 3, 6, 9, 12, 15 and 21 days after the vaccine injection between 9 a.m. and 3 p.m. They were anaesthetized with 2.5 per cent Nembutal sodium (25–50 mg/kg b.w. i.p.). In the non-operated and sham-operated animals the thymus was exposed and the largest of the thymic veins incised. A blood sample was taken in a pipette for supravital staining and for white cell counts.

In all animals the right carotid artery was incised near the origin of the thymic artery and samples of arterial blood were taken for analysis as above. Immediately afterwards the artery was ligated. The influence of the thoracic duct with the left subclavian and jugular veins was then exposed, essentially according to the technique of Reinhardt & Joffey (1957), although the sternum was not incised. The thoracic duct was punctured and a sample of lymph was collected in a pipette for supravital staining. The whole procedure was performed under a dissection microscope. Finally the thymic lobes were removed and weighed and the animals killed. The supravital specimens of blood and lymph were stained with Janus green II and neutral red (see *Frnström & Larsson 1966b*), and were immediately studied under a microscope at 1000 \times magnification. Differential counts were made of lymphocytes with different mitochondrial content. The lymphocytes were registered in six classes: cells with 0, 5, 6, 10, 11–15, 16–20, 21–30 and >30 mitochondria. The average number of mitochondria per lymphocyte was calculated for each sample from the percentages of lymphocytes and the median number of mitochondria in the different classes. For convenience, 0, 10, 11, 20 and >20 mitochondria per cell are denoted as a low, medium and high mitochondrial content respectively. The mitochondrial content (MC) is correlated to the size of the lymphocytes: low MC corresponds to small lymphocytes, high MC to large lymphocytes (*Wiseman 1931; Fichtelius & Larsson 1961; Frnström & Larsson 1963*).

For determination of the absolute number of white cells 25 mm^3 of blood was diluted with 475 mm^3 of *Tissot's* solution (containing methyl violet for staining of white cells). The number of cells was counted in a Bürker counting chamber 285 squares (corresponding to 0.00625 mm^2 each) in each sample. A differential count was made between mono- and polynuclear white cells.

The results were analyzed statistically by Student's *t* test. The comparisons between percentage and number of lymphocytes in thymic vein and carotid artery blood were performed by statistical analysis of the difference in individual animals. The *p* values <0.001 , <0.01 and <0.05 are denoted as highly significant, significant and almost significant respectively.

RESULTS

Operation did not affect the growth of the animals, which was identical in all three groups. No mortality occurred. The mean thymic weight decreased slightly but not significantly after the vaccine injection being minimal 6 days afterwards (-10 per cent).

Antigen Treated Non Operated Animals

A single injection of pertussis vaccine caused a slight increase in the number of lymphocytes in the blood 9–21 days afterwards. The number of granulocytes per mm^3 of blood also increased being maximally raised 9 days after injection. The normal excess of lymphocytes in the thymic vein blood as compared to the carotid artery blood (*Frnström & Larsson 1966a*) persisted after vaccine injection, but no significant increase or decrease in this excess could be demonstrated.

TABLE 1

Comparison Between Differential Counts of Lymphocytes in Carotid Artery and Thymic Vein Blood of Guinea Pigs at Different Intervals after a Single Injection of Pertussis Vaccine Mean \pm S.E.

Source of blood	Days after pertussis vaccination	No. of animals	0-5	6-10	11-15	16-20	21-30	>30	Average number of lymphocytes per cell	p value (reference)
Thymic vein	1	10	22 \pm 0.9	41 \pm 1.0	23 \pm 1.4	8 \pm 0.8	3 \pm 0.3	0 \pm 0	94 \pm 0.1	p < 0.001
Carotid artery	1	10	17 \pm 0.7	37 \pm 1.4	70 \pm 1.6	12 \pm 1.1	4 \pm 0.4	0.2 \pm 0.2	106 \pm 0.2	p < 0.001
Thymic vein	3	10	26 \pm 1.7	47 \pm 1.6	20 \pm 1.5	6 \pm 0.8	2 \pm 0.3	0 \pm 0	86 \pm 0.2	p < 0.001
Carotid artery	3	10	18 \pm 1.4	43 \pm 2.2	26 \pm 1.6	9 \pm 0.7	3 \pm 0.4	0.2 \pm 0.2	99 \pm 0.2	p < 0.001
Thymic vein	6	10	10 \pm 0.8	34 \pm 1.1	33 \pm 1.6	18 \pm 1.0	7 \pm 0.4	0.4 \pm 0.3	121 \pm 0.1	p < 0.001
Carotid artery	6	10	5 \pm 0.6	21 \pm 1.1	38 \pm 1.6	21 \pm 1.6	10 \pm 0.7	2 \pm 0.3	110 \pm 0.3	p < 0.001
Thymic vein	9	10	13 \pm 0.8	42 \pm 1.2	27 \pm 1.7	12 \pm 1.5	5 \pm 0.2	0 \pm 0	105 \pm 0.2	p < 0.001
Carotid artery	9	10	8 \pm 0.8	37 \pm 1.5	36 \pm 1.4	16 \pm 1.5	7 \pm 0.6	0 \pm 0	121 \pm 0.2	p < 0.001
Thymic vein	12	10	26 \pm 1.1	46 \pm 1.0	19 \pm 0.9	6 \pm 0.5	2 \pm 0.5	0 \pm 0	87 \pm 0.1	p < 0.001
Carotid artery	12	10	16 \pm 0.9	19 \pm 1.5	28 \pm 1.7	12 \pm 0.9	4 \pm 0.6	0 \pm 0	106 \pm 0.2	p < 0.001
Thymic vein	15	10	8 \pm 0.5	76 \pm 2.2	70 \pm 1.4	19 \pm 2.3	7 \pm 0.7	0.8 \pm 0.3	123 \pm 0.4	p < 0.01
Carotid artery	18	10	5 \pm 0.4	25 \pm 2.2	34 \pm 1.3	23 \pm 0.9	10 \pm 0.8	2 \pm 0.5	140 \pm 0.2	p < 0.01
Thymic vein	21	10	19 \pm 0.5	43 \pm 0.9	25 \pm 0.9	9 \pm 0.7	5 \pm 0.3	0.2 \pm 0.2	100 \pm 0.1	p < 0.001
Carotid artery	21	10	11 \pm 0.6	36 \pm 1.9	29 \pm 1.2	15 \pm 1.1	7 \pm 0.5	0.8 \pm 0.1	119 \pm 0.3	p < 0.001

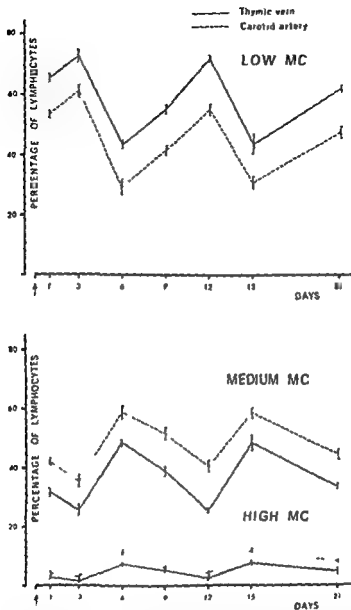


Fig 1

Percentage of lymphocytes with different mitochondrial content (MC) in thymic vein and carotid artery blood of non-operated guinea pigs at different intervals after a single injection of pertussis vaccine

The lymphocytes were subdivided into classes by their mitochondrial content (Table 1). The percentage of blood lymphocytes with different mitochondrial content (MC) changed cyclically during the immunization procedure. Thus the percentage of lymphocytes with low MC (small lymphocytes) had a first maximum 3 days and a second one 12 days after pertussis injection. Concurrently, a corresponding mini-

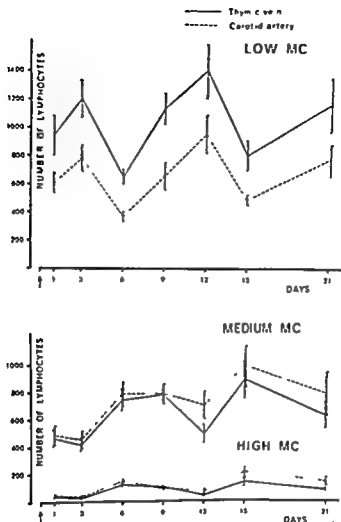


Fig 2

Number of lymphocytes with different mitochondrial content (MC) per mm³ of thymic vein and carotid artery blood of non-operated guinea pigs at different intervals after a single injection of pertussis vaccine

imum was noted in the incidence of lymphocytes with medium and high MC (Fig 1)

Similarly the number of blood lymphocytes with different MC changed cyclically (Fig 2), these changes being significant. The number of "small" lymphocytes per mm³ of blood in the thymic vein, as well as in the carotid artery, decreased from day 3 to day 6 ($p < 0.001$, $p < 0.001$), increased from day 6 to day 12 ($p < 0.001$, $p < 0.01$) and again decreased from day 12 to day 15 ($p < 0.05$, $p < 0.01$). During the whole immunization period, there was an excess of "small" lymphocytes in

TABLE 2

Migration Between Differential Counts of Lymphocytes in Catted Artery Blood of Sham Operated and Thyrectomized Guinea Pigs at Different Intervals after a Single Injection of Pertussis Vaccine Mean \pm S.E.

Days after per- tussis vaccina- tion	No. of mice	0-5	6-10	11-15	16-20	21-30	> 30	Average no. of mito- chondria/ cell	p value of dif- ference
Sham operated	10	16 \pm 0.7	31 \pm 0.7	27 \pm 1.1	12 \pm 1.0	4 \pm 0.2	0 \pm 0	10.4 \pm 0.1	p < 0.001
Thyrectomized	10	8 \pm 0.7	32 \pm 1.9	33 \pm 0.9	19 \pm 0.9	7 \pm 0.7	0.6 \pm 0.3	12.5 \pm 0.3	
Sham operated	10	17 \pm 1.1	40 \pm 1.0	17 \pm 1.0	12 \pm 0.8	1 \pm 0.1	0 \pm 0	10.2 \pm 0.4	p < 0.001
Thyrectomized	10	5 \pm 0.6	17 \pm 2.1	39 \pm 1.5	25 \pm 1.6	10 \pm 0.6	2 \pm 0.4	14.5 \pm 0.1	
Sham operated	10	4 \pm 0.4	18 \pm 0.5	30 \pm 1.2	26 \pm 1.2	10 \pm 0.6	2 \pm 0.6	14.6 \pm 0.1	p < 0.001
Thyrectomized	10	1 \pm 0.4	12 \pm 0.8	37 \pm 1.7	30 \pm 1.5	12 \pm 0.9	5 \pm 0.8	16.6 \pm 0.1	
Sham operated	11	4 \pm 0.5	17 \pm 1.5	37 \pm 1.4	27 \pm 1.4	12 \pm 0.7	3 \pm 0.7	15.3 \pm 0.2	p < 0.001
Thyrectomized	10	0.1 \pm 0.0	10 \pm 0.9	35 \pm 1.1	30 \pm 1.4	20 \pm 0.7	5 \pm 0.7	17.1 \pm 0.2	
Sham operated	12	18 \pm 0.8	40 \pm 0.6	28 \pm 1.0	10 \pm 0.8	4 \pm 0.2	0.4 \pm 0.4	10.3 \pm 0.2	p < 0.001
Thyrectomized	12	5 \pm 0.4	17 \pm 1.0	40 \pm 1.7	23 \pm 1.2	15 \pm 0.9	2 \pm 0.6	15.3 \pm 0.1	
Sham operated	15	5 \pm 0.5	20 \pm 2.2	36 \pm 1.3	26 \pm 1.2	11 \pm 0.3	3 \pm 0.5	14.5 \pm 0.2	p < 0.001
Thyrectomized	15	7 \pm 0.8	20 \pm 0.9	38 \pm 1.4	21 \pm 1.5	11 \pm 0.8	3 \pm 0.5	14.3 \pm 0.2	
Sham operated	21	10 \pm 0.5	32 \pm 0.9	35 \pm 1.1	17 \pm 0.7	8 \pm 0.6	0.4 \pm 0.3	12.3 \pm 0.2	p < 0.001
Thyrectomized	15	8 \pm 0.6	20 \pm 1.0	37 \pm 1.2	23 \pm 1.0	12 \pm 0.8	1 \pm 0.1	14.0 \pm 0.1	

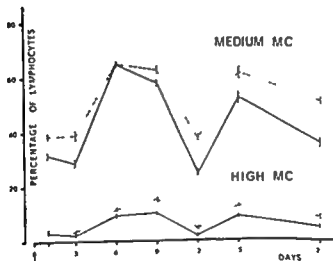
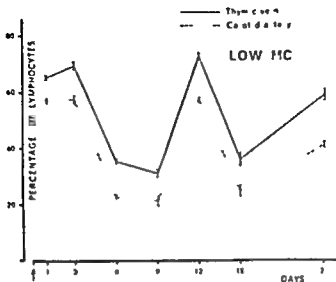


Fig 3

Percentage of lymphocytes with different mitochondrial content (MC) in thymic vein and carotid artery of strain 131 of strain 131 guinea pigs at different intervals after a single injection of pertussis vaccine

the blood from the thymic vein as compared to that from the carotid artery. This excess was also significant ($p < 0.001$ $p < 0.001$ $p < 0.001$ $p < 0.001$ $p < 0.01$ $p < 0.01$ $p < 0.01$ respectively 1 3 6 9 12 15 and 21 days after the vaccine injection). No significant increase or decrease in this excess of small lymphocytes in the thymic vein blood was demonstrable as a result of the antigen injection.

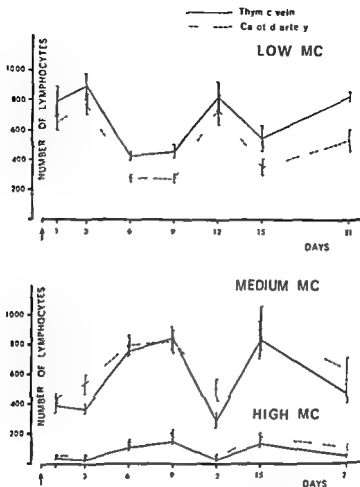


Fig 4

Number of lymphocytes with different mitochondrial content (MC) per mm³ of thymic vein and carotid artery blood of sham operated guinea pigs at different intervals after a single injection of pertussis vaccine

In the thoracic duct lymph the lymphocytes with different MC changed in incidence cyclically in essentially the same way as the blood lymphocytes (Fig 7). Thus the small lymphocytes decreased in incidence from day 3 to day 6 ($p < 0.001$), increased from day 6 to day 12 ($p < 0.001$), again decreased from day 12 to day 15 ($p < 0.001$) and finally increased from day 15 to day 21 ($p < 0.01$).

Antigen Treated Sham Operated Animals

The total number of blood lymphocytes was slightly but not significantly raised 3-21 days after the vaccine injection. The percentage of blood lymphocytes with different MC (Table 2) revealed cyclical changes after administration of the pertussis vaccine essentially as in the identically treated non-operated animals (Fig. 3). The number

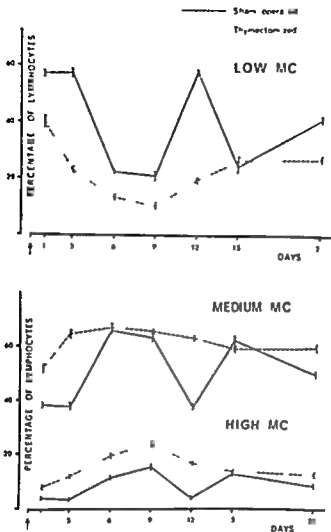


Fig. 5

Percentage of lymphocytes with different mitochrial content (MC) in carotid artery of sham operated and thymectomized guinea pigs at different intervals after a single injection of pertussis vaccine

of blood lymphocytes with different MC also changed in the same way as in the non-operated animals. Thus the number of "small" lymphocytes characterized by a low MC showed two maxima: a first one 3 days after the antigen injection and a second one 12 days after it (Fig. 4). The thymic vein contained more "small" lymphocytes per mm³ of blood than the carotid artery (Fig. 4). This excess of "small" lymphocytes did not change during the immunization procedure. The incidence of different classes of thoracic duct lymphocytes changed essentially as in the non-operated animals (Fig. 7).

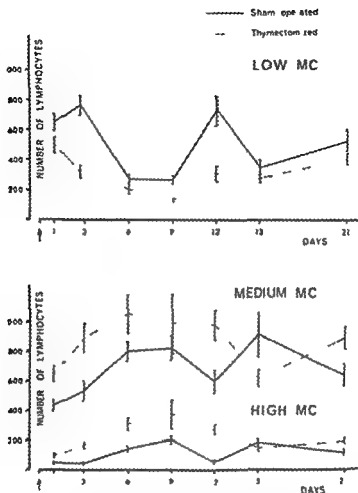


Fig 4

Number of lymphocytes with different mitotic index (MI) per mm³ of ear tip artery blood of sham-operated and thymectomized guinea pigs at different intervals after a single injection of pertussis vaccine.

Antigen Treated Thymectomized Animals

The total number of blood lymphocytes was slightly but not significantly raised 9-12 days after the vaccine injection. The expected changes in the percentage of blood lymphocytes with different MI (Table 2) recorded after injection of pertussis vaccine in non-operated and in sham-operated guinea pigs, did not occur in the thymectomized animals (Fig 5 of Fig 1).

Throughout immunization the thymectomized animals had fewer "small" lymphocytes per mm³ of blood than the corresponding non-operated and sham-operated animals (Fig 6 of Fig 2). The increase in number of small lymphocytes 3 and 12 days after the antigen injection seen in non-operated and sham-operated animals failed to occur in the thymectomized guinea pigs (Fig 6).

TABLE 3

Comparison of Differential Counts of Thoracic-Duct Lymphocytes in Non-Operated, Sham-Operated, and Thymectomized Pigs at Different Intervals after a Single Infection of *Perillus Vaccinae* Mean \pm S.E.

Days after perussina vaccination	No. of animals	Classification of lymphocytes by mitochondrial content						Average no. of mitochondria/cell	p value of difference
		0-5	6-10	11-15	16-20	21-30	>30		
Non-operated	1	5 \pm 0.5	21 \pm 1.6	46 \pm 1.8	19 \pm 1.0	10 \pm 0.4	04 \pm 0.3	13.7 \pm 0.1	p < 0.001
Sham-operated	1	5 \pm 1.7	21 \pm 1.0	42 \pm 0.8	22 \pm 1.1	9 \pm 0.3	09 \pm 0.1	13.9 \pm 0.2	
Thymectomized	1	2 \pm 0.7	14 \pm 1.0	45 \pm 1.8	27 \pm 1.7	11 \pm 0.4	2 \pm 0.4	15.2 \pm 0.2	
Non-operated	3	5 \pm 0.6	23 \pm 0.9	46 \pm 0.8	18 \pm 0.9	8 \pm 0.6	03 \pm 0.2	13.3 \pm 0.1	p < 0.001
Sham-operated	3	5 \pm 0.6	17 \pm 0.8	43 \pm 1.7	24 \pm 1.2	11 \pm 0.5	03 \pm 0.2	14.7 \pm 0.1	
Thymectomized	3	0.2 \pm 0.1	6 \pm 0.4	40 \pm 1.4	33 \pm 1.1	10 \pm 0.6	3 \pm 0.5	17.1 \pm 0.1	
Non-operated	6	1 \pm 0.3	11 \pm 0.1	42 \pm 1.2	29 \pm 1.1	14 \pm 0.8	3 \pm 0.4	16.2 \pm 0.2	p < 0.001
Sham-operated	6	1 \pm 0.3	10 \pm 1.1	42 \pm 1.3	29 \pm 1.4	15 \pm 0.6	3 \pm 0.4	16.3 \pm 0.2	
Thymectomized	6	0 \pm 0	5 \pm 0.6	39 \pm 1.4	35 \pm 1.4	19 \pm 1.0	5 \pm 0.8	17.7 \pm 0.2	
Non-operated	9	2 \pm 0.3	17 \pm 1.2	46 \pm 3.2	24 \pm 1.0	11 \pm 0.9	1 \pm 0.2	14.5 \pm 0.2	p < 0.001
Sham-operated	9	0.8 \pm 0.3	9 \pm 0.7	41 \pm 1.3	30 \pm 1.0	15 \pm 0.5	4 \pm 0.5	16.6 \pm 0.2	
Thymectomized	9	0 \pm 0	6 \pm 0.7	35 \pm 1.5	31 \pm 1.0	22 \pm 1.2	5 \pm 0.8	15.0 \pm 0.2	
Non-operated	12	0 \pm 0.5	24 \pm 0.8	43 \pm 1.5	16 \pm 1.4	9 \pm 0.8	07 \pm 0.3	13.4 \pm 0.2	p < 0.001
Sham-operated	12	3 \pm 0.3	19 \pm 1.2	44 \pm 1.3	24 \pm 1.1	10 \pm 0.5	05 \pm 0.2	14.2 \pm 0.1	
Thymectomized	12	0.2 \pm 0.1	8 \pm 0.9	43 \pm 1.2	30 \pm 1.1	16 \pm 0.8	2 \pm 0.4	16.6 \pm 0.1	
Non-operated	15	0.9 \pm 0.3	12 \pm 0.6	41 \pm 1.2	27 \pm 1.4	14 \pm 0.8	2 \pm 0.3	15.9 \pm 0.2	p < 0.001
Sham-operated	15	1 \pm 0.4	9 \pm 1.0	40 \pm 1.2	32 \pm 1.3	15 \pm 0.8	3 \pm 0.4	16.4 \pm 0.2	
Thymectomized	15	0.8 \pm 0.3	11 \pm 0.9	42 \pm 1.9	29 \pm 1.5	15 \pm 0.5	3 \pm 0.4	16.3 \pm 0.2	
Non-operated	21	3 \pm 0.6	13 \pm 1.3	46 \pm 1.4	24 \pm 1.2	11 \pm 0.6	1 \pm 0.3	14.7 \pm 0.2	p < 0.001
Sham-operated	21	3 \pm 0.3	14 \pm 0.7	42 \pm 1.3	27 \pm 1.2	13 \pm 0.7	2 \pm 0.3	15.2 \pm 0.2	
Thymectomized	21	1 \pm 0.3	10 \pm 0.9	41 \pm 1.1	31 \pm 1.3	14 \pm 0.4	3 \pm 0.5	16.2 \pm 0.2	

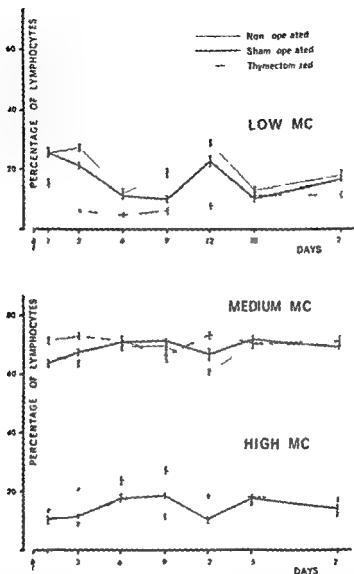


Fig. 7

Percentage of lymphocytes with different mitogenic content (MC) in the cycle test (mg) for non-operated, sham-operated and thyrectomized guinea pigs at different intervals after a single injection of pertussis vaccine.

In the thoracic duct lymph the incidence of "small" lymphocytes was lower than in the guinea pigs with intact thymus (Table 1). The cyclical changes in incidence of "small" lymphocytes observed in the thoracic duct lymph of non-operated and sham-operated guinea pigs after a single injection of pertussis vaccine did not take place in the thyrectomized animals (Fig. 7).

DISCUSSION

The possible participation of the thymus in an immune response to antigenic stimulation has been discussed in several symposia (Good & Gabrielsen 1964, Defendi & Metcalf 1964, Wolstenholme & Porter 1966). In the present study a single subcutaneous injection of pertussis vaccine in a low dose produced no significantly increased or decreased emigration of lymphocytes from the thymus when investigated by direct comparison between afferent and efferent thymic blood. Unexpectedly, the experiment disclosed cyclic changes in incidence and number of different categories of lymphocytes in blood and in thoracic duct lymph and the absence of similar cyclic changes in thymectomized animals.

In normal growing guinea pigs aged 30-50 days the number of lymphocytes with low MC (small lymphocytes) per mm³ of blood increased by about 25 cells a day whereas the number of lymphocytes with medium and high MC was almost constant (Ernst *in* & Larsson 1966b). In the present study on the other hand administration of pertussis vaccine caused a decrease in the number of small circulating blood lymphocytes in both normal and thymectomized animals from 3 to 6 days after injection. Concurrently the larger lymphocytes increased in number. This may have been due to an antigen stimulated transformation of small lymphocytes into larger cells in interrelation which is in accordance with the observation of Hueke *et al.* (1964) (*cf.* phytohemagglutinin induced blastoid transformation of small blood lymphocytes *in vitro*; MacKinney *et al.* 1962, Marshall & Roberts 1963, Sabatin 1966). Another possibility is a disappearance of small lymphocytes from the blood and an independent increase in number of circulating large lymphocytes. In the thymectomized animals the process caused almost total depletion of the smallest lymphocytes (those with 0-1 mitochondria per cell) 9 days after the antigen injection (Table 2). Thus in the non-operated and sham-operated animals the consumption of small lymphocytes seems to have been counteracted by an output of thymic small lymphocytes preventing the total depletion of such cells in the blood.

Between 9 and 12 days after the antigen injection the number of small blood lymphocytes again increased in all groups, this increase being pronounced in the sham-operated and less pronounced in the thymectomized animals. Thus in the non-operated and sham-operated animals these lymphocytes seem to come from both thymus and non-thymic lymphoid tissue.

Following day 12 after the antigen injection a second rapid fall in the number of small lymphocytes and an increase in number of larger lymphocytes occurred in the non-operated and sham-operated animals but not in the thymectomized ones. This second change may have been related to a secondary response to the original antigen injection.

tion and to the generation time of the lymphocytes involved in the immune response (small lymphocytes→large lymphocytes→small lymphocytes→large lymphocytes). Enlargement of small lymphocytes after antigenic stimulation has been reported by *Porter & Cooper* (1962a, b), *Gowan et al.* (1962) and *Rieke et al.* (1963). This implies that the second generation of small lymphocytes may be the cells anamnestic of the original antigen stimulation. The cyclical changes which we observed in the blood lymphocyte population of guinea-pigs vaccinated with the polysaccharide antigen of *B. pertussis* are strikingly similar to the periodical variations in the number of antibody-producing cells in the spleen after immunization with a lipopolysaccharide antigen from *E. coli* (*Britton & Moller* 1966). Our results indicate that most lymphocytes circulating in the blood are involved in immune processes taking place in the organism. It can also be stated that the absence of the thymus interferes with the normal changes in circulating lymphocyte populations occurring after pertussis vaccination.

SUMMARY

Three groups of male guinea-pigs, non-operated, sham-operated and thymectomized, were investigated. The lymphocyte populations of carotid-artery and thymic-vein blood and thoracic-duct lymph were studied at different intervals after a single subcutaneous injection of pertussis vaccine. The lymphocytes were classified by their mitochondrial content.

In the non-operated and sham-operated animals, the number of "small" lymphocytes with 0-10 mitochondria per cell were found to change cyclically, with minima on day 6 and 15 after the antigen injection, when the larger lymphocytes were maximally raised in number. This periodicity did not appear after thymectomy. In the thymectomized animals, the number of "small" lymphocytes was reduced, but tended to be restored at the end of the experimental period.

The thoracic-duct lymphocytes were found to undergo similar changes to those in the blood lymphocytes, although they were less conspicuous.

The normal excess of "small" lymphocytes in the thymic-vein blood as compared to the carotid-artery blood persisted throughout the experiment with no significant increase or decrease.

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BRIEF REPORTS

MIXED CRYOIMMUNOGLOBULINEMIA

By Odd Wager, Jorma A. Räsänen, Allan Lassus and Kaimo A. Mustakallio

By screening techniques cryoglobulins have been found by us in 13 per cent of syphilitic sera (7), in 37 per cent of CBFP sera (4), in 53 per cent of SIF sera (5), in 40 per cent of mononucleosis sera (10) and in 3 per cent of sera from patients with various dermato-venereological diseases (8).

In the present communication some immunological and clinical data of 11 patients with mixed cryoimmunoglobulinemia are reported.

As seen from the table three out of the total of 19 isolated cryoglobulins were composed of IgA and IgG immunoglobulins. Two of the latter contained in addition to IgA and IgG small amounts of β_2A , a moiety of the third component of complement. Immunological studies and case reports of these three patients with IgA IgG cryoglobulinemia will be given elsewhere (9).

All the remaining 16 cryoglobulins were composed of IgM and IgG. One of them also contained β_2A . Four patients had a clinical picture characterized by purpura, arthralgia, Raynaud's phenomenon and rheumatoid factor (RF) activity of their serum (2-3). Three patients had SIF (6). Two were acute nephropathies probably induced by drugs, four patients had syphilis in various stages of the disease and three were cases of mononucleosis. Two of the latter had a typical Paul Bunnell positive infectious mononucleosis, whereas the third was a patient with cytomegalovirus mononucleosis (1).

Most of the isolated cryoglobulins possessed a strong anticomplementary effect, like that shown under the same experimental conditions by known specific precipitates of human and rabbit origin. The anticomplementary activity of the cryoglobulins was abolished by treatment at $+56^\circ\text{C}$ for 30 minutes. Dissociation at pH 3.5 and Sephadex 200 gel filtration of one of the IgM IgG cryoglobulins (11) resulted in separation of the IgM and IgG components, both devoid of cryoprecipitability. Recombination of the two components restored the cryoprecipitability.

The strong anticomplementary activity, the presence of β_2A in three of the mixed cryoglobulins and the dissociation and reprecipitation patterns of one of the cryoglobulins studied suggest that the mixed cryoimmunoglobulins are circulating antigen-antibody complexes. The simultaneous occurrence of a number of recognized autoantibodies (antinuclear antibodies, cold agglutinins, rheumatoid factor activity) in several instances gives further support to this hypothesis.

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THE ORDER OF APPEARANCE OF REACTIVITY TO TREPONEMAL AND LIPOIDAL TESTS IN EARLY SYPHILIS

By A Lassus & A Mustalaino & Aho and T Pullonen

Adequate information of the order of appearance of reactivity to serological test for syphilis can be gained only from untreated syphilitic patients with known date of the infection.

Sera from 76 such male patients—62 with darkfield positive primary and 14 with secondary syphilis—were tested with fluorescent treponemal antibody absorption (FTA ABS) (3) treponema pallidum immobilization (TPI) modified Rafter protein complement fixation (RPCF), Kolmer cholesterol Wassermann (ChWalt) VDRL and Kahn tests. The FTA-ABS test was performed according to the provisional technique published in 1965 by the Venereal Diseases Research Laboratory of the Communicable Diseases Center, Atlanta. The RPCF test was carried out with a microtechnique (2) using 2 units of complement. The other tests were performed according to the US Public Health Service's Manual for Serologic Tests for Syphilis.

In the table the tests are presented in decreasing order of sensitivity in early syphilis. The FTA-ABS test became reactive in practically all cases within 45 days from the infection, the Kolmer and VDRL tests within two months and the other tests three months after infection. Within the first six weeks 6 patients showed reactivity only in the FTA-ABS test and 4 only in some of the lipoidal tests.

TABLE

Appearance of Reactivity to Serological Tests for Syphilis in 76 Cases with Known Date of Infection (Seropositive Cases / Total)

Days from Infection	FTA-ABS	Kolmer	VDRL	Kahn	ChWalt	RPCF	TPI
30	3/11	4/11	1/11	1/11	0/11	0/11	0/11
31-45	18/20	14/20	11/20	10/20	6/20	4/20	2/20
46-60	20/20	19/20	17/20	15/20	11/20	7/20	4/20
61-90	15/15	15/15	14/15	13/15	11/15	8/15*	7/15
91	10/10	10/10	10/10	10/10	10/10	10/10	10/10

* One serum was anticomplementary.

Among the 62 patients with primary syphilis 84 per cent displayed reactivity to FTA-ABS, 77 per cent to Kolmer, 63 per cent to VDRL, 56 per cent to Kahn, 39 per cent to ChWalt, 28 per cent to RPCF, and only 15 per cent to the TPI test. In the 14 cases with secondary syphilis all tests were reactive except the microimmunofluorescent of RPCF, which was negative in two patients.

Deacon and his co-workers (1) have recently reported that the FTA-ABS test gave a positive reaction in 86 per cent of their patients with untreated syphilis, 76 per cent showed reaction to the VDRL, and 63 per cent were reactive in the TPI test. Their patients were not classified according to the duration of syphilitic infection but since a greater proportion of them were reactive to the VDRL test it is probable that the infection was of longer duration on the average in their cases than in our

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cases. This may explain the differences in the percentages of reactive tests being particularly great in the slowest test the TIT.

In conclusion the ITA ABS test detects syphilis earlier than the other treponemal tests and frequently even earlier than the lipoidal tests.

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AUTOIMMUNE PHENOMENA IN PATIENTS WITH OLD TREATED SYPHILIS NONREACTIVE TO THE TREPONEMA TATUM IMMobilIZATION TEST

By A Lassus A A Mustakall and Y Luthren

Syphilitic infection induces immunological aberrations of the autoimmune type including production of antilipoidal reagins (1) of thermic coliform lysine (2) cryoglobulins (3) and rheumatoid factors (4). On the other hand biological false positive (BFP) antilipoidal reagins may develop years before manifestation of certain autoimmune diseases such as systemic lupus erythematosus (SLE) or the rheumatoid arthritis (5). In studies of the chronic BFP phenomenon the fluorescent tre-

ponema has been treated with heavy metals 15 to 53 years ago for either primary or secondary syphilis. Twenty three of the patients were remitted because of severe terminal disease of the following type of syphilis:

In the table the 36 patients are grouped according to their reactivity to the TIT and the lipoidal (STS) test including the VDRL Kahn Venereal Disease Research Laboratory (VDRL) and the Wassermann tests.

Less than 24 out of the 36 patients were nonreactive to the TIT test. Sera from 15 TPI negative cases were available for the ITA ABS test which was positive in 11 cases.

Prevalent autoimmune disease rheumatoid factor activity cryoglobulins were found only among the patients with general reactivity to some of the lipoidal tests (STS + group I and II). In the TIT negative group there was evidence of autoimmune disorder in 8 out of 11 cases and in the TIT positive group in 5 out of 10 cases. In the former group one patient developed definite SLE after the treatment.

Conclusion: In the TPI positive group II definite rheumatoid arthritis developed after syphilis infection in one female patient and cryoglobulinemic purpura in one male patient.

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st rel deeg fr zen f r tw years n the average was available fr m each of the 51 (11P) react rs Nine of these had definite and 7 pr lable systemic lup vers rheumat s s (SLE) adhering to the criteria pre ented elsewhere 3 and 4 definite and 2 pr lable rheumat ll arthritis acc rding t the ARA criteria

Six out of the 9 patients with definite SLE and 2 f the 7 patients with pr lable SLE showed a positive ANF FA test In one of the 2 patients with pr lable SLE the test was positive only with human leucocytes

Among the remaining 25 (11P) react rs showing neither LL-cells nor DNA antibodies 4 had a positive ANF FA test Three f these had probable rheumatoid arthritis They were all females, 34 34 and 67 years old respectively It will be interesting to see whether or not they belong to the rheumat ll variant of SLE (cf 3) The 11 rti ANF FA positive (11P) react r was an asymptomatic 54 year-old man The 4 (11P) reactors with definite rheumat ll arthritis and 2 react rs with pr lable SLE showed a negative ANF FA test

In conclusion the ANF FA test possessed a fairly good discriminatory power for SLE among the 51 (11P) react rs studied

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ANTINUCLEAR FACTORS IN PSORIATIC ARTHRITIS

By A. A. Mustakall, A. Lassus and J. A. Räsänen

The following table shows the results of the ANF FA test in 10 out of 13 patients with psoriatic arthritis. The results are as follows: 10 positive ANF FA test, 3 negative ANF FA test, and 10 positive ANF FA test.

Both cry tal sections from the ant smears of human leucocytes served as the substrates for the indirect ANF FA test (2) The fluorescein conjugated horse anti human globulin serum contained antibodies to human IgG IgM and IgA The psoriatic arthralgias were classified according to antigenological changes

Only on the first and on the second years have the following results been obtained:

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performed using rabbit antisera against pooled normal human γ_2 globulin against the individual undigested M-components and against human light chains. In all cases the results indicated that the fast and slow electrophoretic components consisted of κ and λ Fab fragments respectively. The proportions of λ and κ fragments in the three digests were calculated from light absorbency measurements of eluates from protein stained electrophoresis strips. Determined in this way the quotient λ/κ was 4.2 and was

To visualize the distribution of serological activity on cellular acetate electrophoretograms of M-components and their papain digests blood agar plates containing streptolysin O and α -streptolysin respectively were used. Part of the electrophoresis strips were placed in contact with the plates for a suitable time allowing the proteins to diffuse into the gel and then removed. When AST activity was to be determined the inactive α -streptolysin-O was then activated by application on the plate of a filter paper soaked in p-aminobenzoic acid. Details of this technique will be given elsewhere (Zettervall to be published). Fractions were localized by

material was similarly analysed a maximal inhibition of lysis occurred only at the site of the M-component i.e. nearer the anode than the haemolysis inhibition zone from a digest applied at the same electrophoresis strip.

The patterns were uniformly reproducible and their correspondence to the patterns on the stained gels of the electrophoresis strips was good. These experiments show that the Fab fragments in the three M-components have antihemolytic activity whereas the κ fragments seem inactive.

Studies in progress on the two AST active M-components suggest that streptolysin-O is actually bound by these proteins. Hence the serological activity of these Fab fragments reflects probably also such binding. Heavy and light chains from the M-components seem to be able to react also with

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TRANSIENT MODIFICATION OF THE HELA CELL ORGANELLES FOLLOWING
REDUCTION OF GLUCOSE SUPPLY

By Margrete Willoch *

When the glucose concentration of the nutritive medium of HeLa cells is cut down from the standard 100 mg per cent to 6 mg per cent the submicroscopic morphology of the cell surface is permanently changed in the direction of fewer and less complex cytoplasmic protrusions and smooth cell borders (Willoch 1967). This change is interpreted as a reduction of cell surface area related to a reduced glucose metabolism.

The present note describes the reaction of cytoplasmic organelles to a limitation of accessible glucose.

Material and Methods

HeLa cells strain S3 were kept in the medium E2a of Puck, Cieciura & Fisher (1957) which contains 100 mg per cent glucose. Experiment medium containing 6 mg per cent glucose was prepared according to the same recipe the addition of glucose being omitted and the human serum fraction being treated with baker's yeast and filtered through a bacterial filter before being added to the medium.

Cells were fixed weekly, first time 3 days after transfer to experiment medium. The cultures were fixed in buffered 3 per cent glutaraldehyde, postfixed in buffered 1 per cent OsO_4 and embedded in Vestopal W. Thin sections were cut on an LKB Ultratome and examined in a Zeiss EM 9 electron microscope.

Results

The mitochondria, Golgi region, lysosomes and endoplasmic reticulum of HeLa cells from long term cultures in either of the two media used are all of little specific appearance.

Mitochondria are elongate, occasionally branched, about 0.2 μ microns wide with a rather sparse content of longitudinal or transversal cristae which frequently traverse the total width of a scanty matrix.

The extensive Golgi apparatus consists of several stacks of flat cisternae enclosing a vacuolated area and surrounded by numerous small vesicles.

Lysosomes are most frequently seen in the form of multivesicular bodies.

The endoplasmic reticulum appears as scanty unsystematically arranged short or long profiles without conspicuous granulation.

3 days after transfer to experiment medium the cytoplasmic organelles have the following characteristics.

Mitochondria are of unchanged size. Their matrix is very dense, the cristae being difficult to distinguish.

The vacuolated appearance of the Golgi region is more pronounced owing to a dilatation of the dictyosomal cisternae.

The lysosomes increase markedly in number. They appear as heterogeneously dense bodies mostly containing membrane elements.

There is a strong development of the endoplasmic reticulum which tends to be arranged in relatively dense parallel or whorl-like patterns. Their granulation is markedly increased. The occurrence of granula in the cytoplasm is also more conspicuous.

These structural modifications subside after some 10 days, the cytoplasm regaining the appearance of long term cultures.

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* Fellow of the Norwegian Cancer Society.

Discussion

Being of a purely morphological nature the present observations do not offer the opportunity of an unambiguous functional interpretation.

Transition to experiment medium does not involve detectable changes in long term growth rate or weekly growth pattern of the HeLa cells even immediately after transfer. It thus seems unlikely that the altered organelle transformation represents an effort to maintain a normal reproductive capability.

Whereas a reduction in the glucose supply of HeLa cells induces a corresponding reduction in the total amount of glucose metabolized (Fagle *et al.* 1959) this treatment seems to leave their glycolysis respiratory unit undisturbed (Fagle *et al.* 1959; Thelander *et al.* 1959). The present structural modifications probably indicate a quantitative rather than a qualitative shift in cellular functions.

The involvement of all the organelles suggests that respiratory protein synthesis and digestive processes are all affected by the change of nutritive conditions. The simultaneous appearance and short duration of the transformations also indicate that they are in some way related to each other and that they may represent a shock and functional relaxation reaction to the change in environment.

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LIGAND BINDING SITES FOR STREPTOLYSIN III AND STAPHYLOCOCCAL PROTEIN A ON DIFFERENT PARTS OF THE SAME MYELOMA GLOBULIN

by Gösta Kronwall

Dammare & Clausen (1966) reported an IgG myeloma globulin preparation precipitating staphylococcal protein A as well as an antigen from *Haemophilus pneumoniae* type 4. Dammare & Clausen were apparently inclined to ascribe the anti-protein A activity to the contaminating normal gamma globulin in the myeloma globulin preparation.

I have tested 44 myeloma sera containing IgG myeloma globulins for anti-streptolysin O and for precipitation of protein A (Löfgren & Sjögren 1963; Löfgren 1966) from *Staph aureus* T₂ sera showed exceptually high titres of anti-streptolysin O (AST 280 000 and 70 000 IU per ml respectively). On immunoelectrophoresis these 2 myeloma sera precipitated protein A like several of the other myeloma sera with the formation of an arc referring to the myeloma band. The gamma globulin concentrations of myeloma serum dilutions still giving a precipitate against protein A in agar gel diffusion tests were lower than that of a pool of normal human sera. Serum from patient M.P. with an AST titre of 280 000 IU per ml was investigated further.

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2 Purified protein A was kindly supplied by Dr T. Löfgren, Lund.

The myeloma globulin present in the serum M P at a concentration of 5.3 g per 100 ml was typed Gm(-1 -24 -5) (nomenclature according to WHO 1963) with light chains of λ type. The myeloma globulin was purified in two steps by gel filtration and zone electrophoresis (Kilander 1963). On immunoelectrophoresis the preparation showed no traces of normal gamma globulin when tested at 2.9 g per 100 ml. Papain digestion and separation of the fragment were performed according to Porter (1959). Heavy and light chains were prepared and separated according to Olins & Feldman (1964).

The AST titres of the purified myeloma globulin and of the Fab fragment was about 22 000 IU per ml at a concentration of 10 mg protein per ml. The corresponding AST titre of the light chain preparation was 1700 IU per ml whereas the Fab fragment and the preparation of heavy chains did not show any significant activity. The streptolysin O combining site has been located on the Fab fragment for two other myeloma globulins with high AST activity (Zettervall unpublished work).

On immunoelectrophoresis the myeloma globulin preparation like the original serum precipitated purified protein A from *Staph aureus* with an arc suggesting a monoclonal precipitating antibody as described earlier by Damasco & Clausen (1966). In the recent study by Forsgren & Sjöquist (1966) the combining site for staphylococcal protein A as shown by inhibition experiments was said to be located on the heavy chains and on the Fab fragments of normal human gamma globulin and myeloma globulins. Precipitation of protein A by the purified myeloma globulin M P was inhibited by the heavy chains but not by the light chains or by the Fab and Fc fragments. The papain digestion technique resulted in a more extensive degradation than was to be expected. This may explain why the preparation of the protein A combining site is presumably in that part which is broken down to small fragments after papain digestion.

According to Jensen (1959) the agglutination of *Staph aureus* by normal human sera is an effect of the combination between protein A on the surface of the cocci and corresponding antibodies. To ascertain whether the combining sites for streptolysin O and protein A were located on the same molecules or whether the myeloma globulin was heterogeneous in this respect absorption by living staphylococci was performed. Staphylococci Cowan type 1 phage type 52 from an 18 hour culture grown on solid medium were washed in saline. 4×10^{12} bacteria were added to 1 ml of the myeloma globulin and the Fab fragment preparation containing 50 µg protein respectively. After 2 hours at room temperature the tubes were centrifuged and the supernatants tested for AST activity. Controls without added bacteria were included.

AST Titre of Purified Myeloma Globulin and Fab Fragment after Absorption by *Staph aureus*. The Titre is Expressed as IU per ml at a Protein Concentration of 10 mg per ml

Myeloma globulin		Fab fragment	
absorbed	not absorbed	absorbed	not absorbed
400	22 000	11 200	17 000

As shown in the table the AST activity of the purified myeloma globulin was reduced to less than 2 per cent after absorption by staphylococci whereas the AST activity of the Fab fragment was only slightly reduced.

These absorption experiments indicate that ligand binding sites for streptolysin O and staphylococcal protein A are located on different parts of the same myeloma globulin molecules. These immunoglobulin molecules may thus react with streptolysin O and with protein A although the latter proteins do not react in the ordinary sense of this term. At least some immunoglobulin molecules may thus have complementary sites for more than one structure in this class both structures being of bacterial origin.

The myeloma globulin present in the serum M P at a concentration of 5.3 g per 100 ml was typed Gm(-) — 2.4 — 5) (nomenclature according to WHO 1960) with light chains of λ type. The myeloma globulin was purified in two steps by gel filtration and zone electrophoresis (Kallander 1963). On immunoelectrophoresis the preparation showed no traces of normal gamma globulin when tested at 2.9 g per 100 ml. Papain digestion and separation of the fragment were performed according to Porter (1959). Heavy and light chains were prepared and separated according to Olins & Edelman (1964).

The AST titres of the purified myeloma globulin and Fab fragment were as follows:

The purified protein A from *Staph aureus* has an active site suggesting ammanno & Clausen combining site for heavy chains and on the Fc fragments of normal human gamma globulin and myeloma globulins. Precipitation of protein A by the purified myeloma globulin M P was inhibited by the heavy chains but not by the light chains or by the Fab and Fc fragments. The papain digestion technique resulted in a more extensive degradation than what is generally reported (Kronvall 1965) and this may explain why the preparation of Fc fragments did not show any inhibition. Thus the protein A combining site of myeloma globulin M P resides in the heavy chains presumably in that part which is broken down to small peptides by prolonged papain digestion.

According to Jensen (1959) the agglutination of *Staph aureus* by normal human sera is an effect of the combination between protein A on the surface of the cocci and corresponding antibodies. To ascertain whether the combining sites for streptolysin II and protein A were located on the same molecules or whether the myeloma globulin

was performed in a culture grown to 1 ml of the 10^8 protein suspension. After 2 hours at room temperature the tubes were centrifuged and the supernatants tested for AST activity. Controls without added bacteria were included.

AST Titre of Purified Myeloma Globulin and Fab Fragment after Absorption by *Staph aureus*. The Titre is Expressed as IU per ml at a Protein Concentration of 10 mg per ml

Myeloma globulin		Fab fragment	
absorbed	not absorbed	absorbed	not absorbed
400	22 000	11 200	17 600

As shown in the table the AST activity of the purified myeloma globulin was reduced to less than 2 per cent after absorption by staphylococci whereas the AST activity of the Fab fragment was only slightly reduced.

These absorption experiments indicate that the active site for streptolysin II is not the same as the active site for protein A. The same myeloma globulin reacts with streptolysin II and protein A, and cross reacts in the immunodiffusion test. At least some immunoglobulin G molecules may thus have complementary sites for more than one structure in this study both structures being of bacterial origin.

TABLE 1

Data about the Examined Animals the Tracers Used and a Summary of the Results (+ indicates areas in which the blood vessels allow the passage of the tracer - the opposite)

Species	Number	Body weight (grams)	Tracer	Circulation time (hrs)	Permeability of vasa nervorum	
					Endoneurium	Epineurium
Rat	25	150-350	FLA, EBA	1/4-24	—	+
Guinea pig	9	315-460	FLA, FBA	1/4 6	+	+
Rabbit	5	1400-1600	FLA, FBA	2 6	+	+
Hen	4	1300-1900	EBA	2-6	+	+
Cat	4	1300-2700	FLA	5/60 24	•	+

* The two cats examined five minutes after the injection did not show signs of albumin extravasation contrary to the other two cats examined twenty four hours later

were killed at various times thereafter and the two sciatic nerves were removed for examination. The details of this technique have previously been described (Olsson 1966)

Results

Subsequent to injection of either FLA or FBA an intense fluorescence was visible in the lumen of vasa nervorum of all animals. An extravasation of labelled was also observed both in the epineurium and the perineurium. Here an infiltration of albumin increasing with time after injection suggestive of a slow transvascular passage of albumin was observed.

There were obvious species differences in the vascular permeability to serum albumin in the endoneurium of the nerves. Whereas the rats did not show transvascular passage of the fluorescent albumin in the endoneurium (Fig 1) this was regularly observed in rabbits, guinea pigs, hens and cats when the animals were allowed

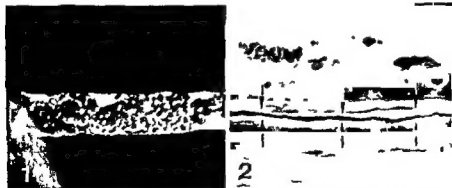


Fig 1 Fluorescent albumin confined to the lumen of an endoneurial blood vessel of a rat sciatic nerve. There is no fluorescence outside the blood vessel.

Fig 2 Longitudinal section through a guinea pig nerve after intravenous injection of FLA. Note the cellular uptake and the diffuse extravascular fluorescence of FLA in the epineurium (upper part); the accumulation of FLA in the perineurium (upper part) and the diffuse faint fluorescence in the endoneurium (lower part) with accentuation in the subperineurial space (lower part).

to survive for six hours or more after the injection (Fig. 2). In these animals varying amounts of fluorescent albumin were present in the endoneurial interspaces. The penetration rates seemed also to differ in animals in the latter group, but the present material is insufficient to provide quantitative aspects on this problem.

An interesting finding frequently encountered in rabbits, guinea pigs and dogs was the accumulation of fluorescent albumin in a narrow zone immediately beneath the perineurial epithelium (Fig. 2). This finding proves that the subperineurial space often seen in histological sections is not exclusively an artefact but a preformed tissue space in connection with the endoneurial spaces.

Discussion

The present findings demonstrate that the permeability of vasa nervorum to serum albumin shows species variations. Previously Wakman (1961) observed in a study on the permeability of vasa nervorum to dipterocytin and various dyes that also the permeability to these substances showed species differences. He considered the permeability properties of vasa nervorum to be of considerable significance for determination of the distribution of nerve fibre lesions in peripheral neuropathies caused by bloodborne agents. Wakman was of the opinion that the reason for the resistance of rabbit peripheral nerves to experimental dipterocytin might be that the circulating toxin cannot pass from the blood into the endoneurium of rabbit peripheral nerves. However, the present study has shown that the vasa nervorum of normal rabbit nerves permit the passage of albumin across their walls. One reason for these diverging results might be that dipterocytin and albumin penetrate at different rates across the vascular walls. Obviously this must be the object of further investigations.

Since intravenously injected fluorescent albumin can enter into the endoneurial compartment of peripheral nerves in normal rats this species is well suited for further experiments on the behaviour of vascular permeability in experimentally induced peripheral neuropathies since even small differences can be detected. However, when the other species tested in the present study take to be used the normally occurring endoneurial infiltration of serum albumin must be taken into consideration.

Summary

A preliminary study of the vascular permeability of serum albumin of peripheral nerves in different species is reported. The results show that species differences in the permeability of endoneurial blood vessels to circulating serum albumin exist in the peripheral nerves.

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